393

Attorney Docket No. 5800-8A (35800/185816) PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

#19
B.GJ
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In re:	Glucksmann *et al.*	Confirmation No.	6988
Appl. No.:	09/383,745	Group Art Unit:	1646
Filed:	August 26, 1999	Examiner:	E. Lazar Wesley
For:	14926 RECEPTOR, A NOVEL G-PROTEIN COUPLED RECEPTOR		

June 21, 2002

Commissioner for Patents
Washington, DC 20231

APPEAL BRIEF

Sir:

This Appeal Brief is filed pursuant to the "Notice of Appeal to the Board of Patent Appeals and Interferences" mailed April 12, 2002 and received by the Office on April 22, 2002.

Real Party in Interest.

The real party in interest in this appeal is Millennium Pharmaceuticals, Inc., the assignee of the above-referenced patent application.

Related Appeals and Interferences.

U.S. Patent Application serial number 09/145,745, filed September 2, 1998, which is the parent case for the present application, is currently on appeal to the Board of Patent Appeals and Interferences.

Status of Claims.

Claims 32-59 are the subject of this appeal. The claims appear in Appendix A. Claims 1-32 have been cancelled.

Status of Amendments.

The Examiner has indicated that the Amendment After Final Rejection mailed March 13, 2002 will be entered upon the timely submission of an Appeal Brief and the requisite fees.

Summary of the Invention.

The pending claims of the present invention are directed to methods of modulating the activity of G-protein coupled receptor (GPCR) comprising the amino acid sequence set forth in SEQ ID NO:1, the amino acid sequence set forth as amino acids 6 to 370 of the amino acid sequence set forth in SEQ ID NO:1, or the amino acid sequence a sequence variant of the amino acid sequence set forth in SEQ ID NO:1, where the variant has G-protein mediated signal transduction activity, and to methods of identifying a compound that modulates the activity of a polypeptide comprising the amino acid sequence set forth in SEQ ID NO:1, the amino acid sequence set forth as amino acids 6 to 370 of the amino acid sequence set forth in SEQ ID NO:1, or the amino acid sequence a variant of the amino acid sequence set forth in SEQ ID NO:1, where the variant has G-protein mediated signal transduction activity. The 14926 receptor is a member of a family of proteins that are known in the art for their importance as therapeutic targets.

Issues.

Issue 1--Whether the invention of claims 32-59 has utility under 35 U.S.C. §101 and thus is enabled under 35 U.S.C. §112, first paragraph.

While the Examiner has rejected the claims under 35 U.S.C. §101 and 35 U.S.C. §112, first paragraph, both rejections hinge on whether Applicant has established a utility for the 14926 receptor. In rejecting claim 32-59 under 35 U.S.C. §112, first paragraph, in the final Office Action, the Examiner states, "since the claimed invention is not supported by either a specific asserted utility or a well established utility for the reasons set forth above, one skilled in the art clearly would not know how to use the claimed invention." December 14, 2001 Office Action,

page 6. Therefore, both rejections are based on the fact that the Examiner has not accepted the evidence that the14926 polypeptide has patentable utility.

Issue 2—Whether the invention of claims 52-59 is enabled under 35 U.S.C. § 112, first paragraph.

Issue 3--Whether the invention of claims 37-46 and 54-57 meets the written description requirement set forth in 35 U.S.C. § 112, first paragraph.

Grouping of Claims.

The claims do not stand or fall together. While all the pending claims have been rejected under 35 U.S.C. § 101, claims 52-59 have also been rejected under 35 U.S.C. § 112, first paragraph on the grounds that they are not enabled and claims 37-46 and 54-57 have been rejected under 35 U.S.C. § 112, first paragraph, on the grounds that the specification does not provide adequate support for variants of the 14926 polypeptides, while claims 32-36, 47-53, 58, and 59 do not recite such variants. Accordingly, the issues surrounding the claims are different, and the claims do not stand or fall together.

Argument.

Issue 1--Whether the invention of claims 32-59 has utility under 35 U.S.C. §101 and thus is enabled under 35 U.S.C. §112, first paragraph.

The Examiner has rejected claims 32-59 under 35 U.S.C. §101 as lacking patentable utility and as lacking enablement under 35 U.S.C. §112, first paragraph. The Examiner states, "[a]pplicants do not provide a specific and well established utility providing patentability for their invention. The invention therefore does not fulfill the requirements of 35 USC 101." (December 14, 2001 Office Action, page 3) In fact, Applicants have asserted a utility for the

claimed invention that is specific, substantial, and credible and therefore meets the requirements for patentability.

I. 14926 Has Specific, Substantial, and Credible Utility

The pending claims of the instant invention are drawn to methods of modulating the activity of a G-protein coupled receptor and methods of identifying a compound that modulates the activity of a G-protein coupled receptor. In the Office Action mailed December 14, 2001 (paper number 14), the Examiner states that the claims are not rejected "under the grounds that 14926 might be a G-protein coupled receptor . . . but rather than at the time the application was filed, appellants have not provided a specific and substantial utility for the gene." December 14, 2001 Office Action, page 4. The Examiner further states, "appellants do not provide a specific utility for the claimed '14926 receptor', as for example no ligand for the receptor and no specific function for the receptor are disclosed." December 14, 2001 Office Action, page 3.

Accordingly, the rejection under 35 U.S.C. § 101 for lack of patentable utility is based on the premise that no utility asserted by the Applicant can be specific and substantial unless it depends in some way on the endogenous ligand or the physiologic function of the 14926 G-protein coupled receptor, and therefore the Examiner's burden of establishing a *prima facie* case of lack of utility may be met by demonstrating that the Applicant has not provided the endogenous ligand or physiologic function of the claimed receptor nucleotide sequence.

In fact, the Applicant has asserted specific, substantial utilities for the claimed invention that do not require a knowledge of either the endogenous ligand or the precise biological function of the 14926 receptor in order to be operable. The utilities asserted for the 14926 receptor are based upon the unique properties of G-protein coupled receptors, including their modulation by small molecules, their signal transduction activity, and their resulting role as important therapeutic targets. These asserted utilities meet the requirements set forth in the statute, the applicable case law, and the utility examination guidelines.

A. Those of skill in the art recognize the utility of G-protein coupled receptors

The Examiner has rejected claims 32-59 under 35 U.S.C. §101 on the grounds that the claimed invention "requires further research to have a specific and substantial utility." (December 14, 2001, Office Action, page 4). This does not correctly reflect the view of orphan G-protein coupled receptors in the art, where it is known that "[h]istorically, the superfamily of GPCRs has proven to be among the most successful drug targets and consequently these newly isolated orphan receptors have great potential for pioneer drug discovery" (Stadel *et al.* (1997) *Trends Pharmacol. Sci.* 18:430-436; provided as Appendix B). Accordingly, those of skill in the art recognize that the identification of an orphan G-protein coupled receptor provides an immediate benefit.

1. Novel GPCRs are useful as members of selectivity screening panels.

Those of skill in the art recognize that the identification of a novel member of the G-protein coupled receptor family provides an immediate benefit because all members of the GPCR protein family have utility in selectivity screening of candidate drugs that target GPCRs. It is known in the art that the clinical usefulness of a therapeutic compound is determined not only by its ability to bind and modulate a molecular target of interest, but also by its selectivity. Drugs that bind selectively to their molecular target are highly preferred over those that bind to structurally related molecules, as the selective compounds are far less likely to have unwanted side effects in clinical use. Thus, an important component of any drug development strategy is determining the selectivity of the candidate drug for the molecular target of interest over structurally related polypeptides.

2. The usefulness of members of a selectivity screening panel is not dependent upon their in vivo physiological role or endogenous ligand.

The effectiveness of selectivity screening increases in proportion with the number of structurally related polypeptides screened. Furthermore, it is the interaction between the candidate drug and each panel member that is the focus of selectivity screening. Thus, the

usefulness of these structurally related polypeptides (such as Applicant's) is not dependent on their biological role or ligand-binding properties; their utility comes from the fact that they share significant sequence identity with the molecular target of the candidate drug.

3. The art teaches the use of orphan receptors in selectivity screening.

Applicant have previously provided an example of the use of orphan receptors in selectivity screening: Goodwin *et al.* (2000) *Molecular Cell* 6:517-526 (**Appendix C**). This reference describes the use of a panel of structurally-related nuclear receptors to identify a specific agonist for FXR, a nuclear receptor that regulates bile acid synthesis and is a target in the treatment of cholestasis. The authors state that many previously-identified FXR ligands interact with other proteins including bile-acid-binding proteins and transporters (Goodwin *et al., ibid.*, page 518, column 1, first full paragraph). In order to identify a compound that selectively modulates FXR, the authors of Goodwin *et al.* screened for compounds that modulated FXR activity and then tested these compounds for their ability to activate *other* nuclear receptors that share structural similarity with FXR. Figure 1C of Goodwin *et al.* shows that the compound GW4064 potently activates FXR but does not modulate the activity of the other nuclear receptors tested. Note that the nuclear receptor panel screened in Figure 1C includes the orphan nuclear receptors SHP-1 and LRH-1 in addition to receptors having previously-identified ligands. Thus, the orphan receptors SHP-1 and LRH-1 derive their utility as members of a selectivity screening panel based on their sequence similarity with a receptor of therapeutic importance.

4. The identification of the 14926 ligand is not a requirement for establishing the utility of this receptor in drug screening.

The Examiner has repeatedly stated that the Applicant must provide the 14926 ligand or 14926 cellular function in order to establish the utility claims. The final Office Action states, "applicants do not provide a specific utility for the claimed '14926 receptor', as for example no ligand for the receptor and no specific function for the receptor are disclosed." (December 14,

2001 Office Action, page 4). While this statement may reflect the thinking of the pre-genomics era, it does not accurately describe the current state of the art in drug discovery.

Those of skill in the art appreciate that rapid advances in technology have led to dramatic changes in the way in which research is conducted in many biomedical-related areas. "Molecular biology has had a dramatic influence" on active drug discovery and research projects in the pharmaceutical industry, particularly those involving GPCRs. Stadel *et al.* (1997) *Trends Pharmacol. Sci.* 18:430-436; provided as Appendix B. These advances in molecular biology have led to what those in the art consider a "paradigm shift" in the way research and drug discovery is conducted. *Id.*

In the new drug discovery paradigm, the starting point in the process is the identification of new members of gene families such as the GPCR superfamily by "computational or bioinformatic methodologies." Stadel *et al.* at 430. "Once new members of the GPCR superfamily are identified, the recombinantly expressed receptors are used in functional assays to search for the associated novel ligands. The receptor-ligand pair are then used for compound bank screening to identify a lead compound that, together with the activating ligand, is used for biological and pathophysiological studies to determine the function and potential therapeutic value of a receptor antagonist (or agonist) in ameliorating a disease process." Stadel *et al.* at 434. Thus, in the reverse molecular pharmacology approach to drug development, it is the a full-length cloned receptor, rather than a ligand having an unknown molecular target, that is the starting point of the drug discovery process.

The Examiner has argued that Applicant's arguments demonstrating that those of skill in the art consider orphan receptors to have utility are unpersuasive and quotes from the last paragraph of the first column of page 434 of Stadel *et al.*, where the authors state, "[t]he reverse molecular pharmacology strategy is a far more daunting challenge and risky endeavor when compared with the more traditional approach, since the starting material for a drug discovery effort is simply an orphan receptor of unknown function, with no apparent relationship to a disease indication." To put this statement in its proper context, however, one must consider the very next sentence of Stadel *et al.*, in which the authors state:

> the potential reward of using this approach is that resultant drugs naturally will be pioneer or innovative discoveries, and a significant proportion of these unique drugs may be useful to treat diseases for which existing therapies are lacking or insufficient.

Stadel *et al* at 434. Furthermore, when the Stadel *et al.* reference is considered in its entirety, it is clear that its primary teaching is not that the process of discovering drugs using a reverse molecular pharmacological approach is insurmountably difficult and should not be attempted, but rather that the reverse molecular pharmacological approach described is already being actively pursued because "the pharmaceutical industry has recognized the power of genomics to provide it with new and unique drug targets." Stadel *et al.* at 436. Accordingly, this reference provides evidence that those of skill in the art recognize the real-world utility of novel orphan GPCRs.

B. GPCRs share a specific, substantial, and credible utility.

In response to the evidence that Applicant has presented to demonstrate that the 14926 receptor has real-world utility, the Examiner has argued that the use of the 14926 receptor sequence in drug screening and selectivity screening is not a specific utility because "it does not rely on a particular characteristic of the instant 14926 gene, but rather relies on features shared by many diverse GPCRs." (December 14, 2001 Office Action, page 5). This argument is at odds with the "Utility Examination Guidelines," which provide that "[w]hen a class of proteins is defined such that the members share a specific, substantial, and credible utility, the reasonable assignment of a new protein to the class of sufficiently conserved proteins would impute the same specific, substantial, and credible utility to the assigned protein." 66 Fed. Reg. 1092, 1097 (2001). This statement from the "Utility Examination Guidelines" makes it clear that when a sequence is shown to encode a polypeptide belonging to a family of proteins that share a common utility, this supports the conclusion that the novel sequence has specific utility.

As further evidence that sequence identity with a class of proteins having a specific and substantial utility may be used to establish the specific and substantial utility of a polypeptide, Applicant cites Example 10 of the "Revised Interim Utility Guidelines Training Materials."

Example 10 is directed to a nucleic acid encoding a polypeptide having a high level of sequence identity with DNA ligases. If the policy set forth in the Examiner's Answer as described above were followed, the polypeptide claimed in Example 10 of the "Revised Interim Utility Guidelines Training Materials" would be rejected for lack of utility because the well-established utility in this example is based on the claimed polypeptide's ligase activity and this utility is shared with all members of the ligase family of proteins. Instead, however, it is concluded in the analysis of this example that the claimed ligase has patentable utility. The patentable utility is demonstrated *because* the ligase can be used for the same purpose as other members of the ligase family of proteins, not in spite of this fact.

Similarly, Applicant has shown that the 14926 receptor is a member of the rhodopsin subfamily of GPCRs. This family of receptors bind small molecules and mediate signal transduction via phosphatidylinositol-mediated pathways or cyclic AMP-mediated pathways, and these unique properties have historically made GPCRs among the most successful drug targets. *See*, Stadel *et al.* (1997) *Trends Pharmacological Science* 18 at 436. In fact, the 14926 receptor belongs to a family of receptors that are the target for more than 50% of all prescription drugs. Attwood (2001) *Trends Pharmacological Science* 22:162-65. Accordingly, the utilities asserted for the claimed 14926 GPCR in drug screening and selectivity screening are based on the unique properties shared by the rhodopsin family of GPCRs.

Example 10 of the "Revised Interim Utility Guidelines Training Materials" also demonstrates that the establishment of patentable utility does not require that the endogenous substrates and physiologic role of a polypeptide be known if the asserted or well-established utilities are operable without this knowledge. In Example 10, the well-established utility of the claimed polypeptide is based on its *in vitro* biochemical activity. According to the analysis in the example, "DNA ligases have a well-established use in the molecular biology art based on this class of protein's ability to ligate DNA ("Revised Interim Utility Guidelines Training Materials," March 1, 2000, page 54). Thus, because the utility of the claimed ligase nucleotide sequence is well-established in molecular biology, the endogenous substrates and biological role of the ligase in the cells in which it is expressed are not required to establish utility. Similarly, the well-

established utility of the 14926 GPCR in drug development is based on its biochemical activity, *i.e.* its G-protein-mediated signal transduction activity. Just as the biochemical activity of the ligase of Example 10 confers patentable utility to this protein in the absence of its physiologic substrates or utility, the biochemical activity of the 14926 receptor confers patentable utility to this receptor in the absence of its specific ligands or physiologic function.

Accordingly, the Examiner's arguments in support of a *prima facie* case of lack of utility are based on the premise that the endogenous ligand, specific function, or disease association of the 14926 receptor is an absolute requirement for the establishment of specific utility. Applicant has asserted utilities for the claimed receptor sequences that do not depend on the endogenous ligand or function of the receptor and has provided evidence from the scientific literature that demonstrates that the asserted utilities are specific, substantial, and credible. Nevertheless, the Examiner has maintained the rejection of the claims under 35 U.S.C. § 101.

C. The *Manual of Patent Examination Procedure* provides that claims should not be rejected for lack of patentability utility because the claimed invention is to be used in a research setting.

The Examiner argues that utilities asserted by the Applicants "constitute an invitation to experiment and an invitation to use the gene and encoded protein as a research tool." (December 14, Office Action, pages 4 and 5). The argument that an asserted utility as a research tool is not sufficient to satisfy the utility requirement of 35 U.S.C. § 101 is contrary to the provisions set forth in the eighth edition of the *Manual of Patent Examination Procedure*, which states that inventions should not be rejected for lack of utility merely because they are to be used in a research or laboratory setting. The *Manual of Patent Examination Procedure* provides:

> confusion can result when one attempts to label certain types of inventions as not being capable of having a specific and substantial utility based on the setting in which the invention is to be used. One example is inventions to be used in a research or laboratory setting. Many research tools such as gas chromatographs, *screening assays*, and nucleotide sequencing techniques have a clear, specific, and unquestionable utility (e.g. they are useful in analyzing compounds). An assessment that focuses on whether an invention is useful only in a research

> setting thus does not address whether the invention is in fact "useful" in a patent sense.

Manual of Patent Examination Procedure § 2107.01 (8th ed. 2001), emphasis added.

Accordingly, Applicant's asserted utilities for the 14926 receptor in drug screening and selectivity screening are not insubstantial or non-specific merely because these utilities are operable in a laboratory setting. Furthermore, no additional research is required to confirm that the 14926 receptor has utility in drug screening and selectivity screening. Applicants have demonstrated that the 14926 receptor is a member of the rhodopsin family of GPCRs, a family of cell membrane receptors that bind small molecules to mediate signal transduction pathways and have historically been among the most successful drug targets. Because of these properties, which are unique to rhodopsin family G-protein coupled receptors, those of skill in the art recognize that the identification of novel orphan rhodopsin family G-protein coupled receptors has real-world value in the pharmaceutical research field as a tool in drug screening and selectivity screening, even in the absence of experimental evidence demonstrating the 14926 receptor ligand or physiologic function.

II. A prima facie showing of no utility has not been presented.

The "Examination Guidelines for the Utility Requirement" (MPEP § 2107) set forth the elements required to establish a *prima facie* case of no utility as follows:

> Where the asserted utility is not specific or substantial, a *prima facie* showing must establish that it is more likely than not that a person of ordinary skill in the art would not consider that any utility asserted by the applicant would be specific and substantial. The prima facie showing must contain the following elements:
>
> (i) An explanation that clearly sets forth the reasoning used in concluding that the asserted utility for the claimed invention is not both specific and substantial nor well-established;
>
> (ii) Support for factual findings relied upon in reaching this conclusion; and

(iii) An evaluation of all relevant evidence of record, including utilities taught in the closest prior art.

MPEP § 2107. This is in accordance with *In re Brana*, 34 U.S.P.Q.2d 1437, 1441 (Fed. Cir. 1995), where the Federal Circuit held that, "[o]nly after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the Applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility.")

In the present case, Applicants have demonstrated that the 14926 receptor functions as a G-protein coupled receptor and that the claimed 14926 nucleotide sequences are useful in drug screening, including screening for compounds that modulate 14926 signal transduction activity and selectivity screening for candidate drugs that specifically target GPCRs that share sequence similarity with the 14926 receptor.

Nevertheless, the Examiner has maintained the rejection under 35 U.S.C. § 101. The premise underlying the Examiner's rejection is that a person of ordinary skill in the art would not consider the use of an orphan GPCR in drug screening to be useful in the absence of the 14926 ligand and physiologic function or a specific disease associated with the 14926 receptor, and that further research is required to demonstrate the patentable utility of this receptor. However, contrary to the requirements of MPEP § 2107, the Examiner has not provided the factual findings or evidence relied on in reaching this conclusion.

Furthermore, Applicant has provided evidence demonstrating the specific and substantial utility of orphan receptors in the drug screening process. As described above, Applicants have provided evidence showing that orphan receptors play a critical role in identifying agonists and antagonists that bind to therapeutic targets but not to structurally-related molecules. Applicants have also provided evidence showing that because the rhodopsin family of G-protein coupled receptors contains a number of key drug targets, members of this family share a common real world use in drug screening including selectivity screening of drugs. For example, the rhodopsin family of GPCRs includes targets for the treatment of numerous

disorders including depression, anxiety, migraine, asthma, hypertension, and other cardiovascular disorders. Accordingly, the 14926 receptor has a specific, substantial, and credible utility in selectivity screening for drugs targeting rhodopsin family GPCRs. The utility of the 14926 receptor is not dependent on its biological role or ligand-binding properties, rather the utility results from its sequence similarity with drug targets in the rhodopsin family of GPCRs.

The Examiner has stated that "applicants do not provide a specific utility for the claimed '14926 receptor', as for example no ligand for the receptor and no specific function for the receptor are disclosed." (December 14, 2001 Office Action, page 3). However, the Examiner has not provided any language from the Utility Guidelines or case law to support the assertion that a knowledge the ligand and physiologic function of a receptor is a *per se* requirement for the establishment of patentability utility of the 14926 receptor, even when applicants have asserted a specific, substantial, and credible utility that does not depend on this precise physiologic function. Furthermore, the Examiner has not provided any factual findings or evidence to demonstrate that a knowledge of the precise physiologic function of the receptor is required in order to use the invention in the manner asserted by the Applicants, or that a person of ordinary skill in the art would find that the claimed invention lacked specific and substantial utility in selectivity screening and drug screening in the absence of a knowledge the 14926 receptor's precise physiologic function. Similarly, the Examiner argues that the utility asserted by the Applicant is "a starting point or a hint for further research," (December 14, 2001 Office Action, page 4) but does not provide factual findings or evidence describing the nature of the additional research alleged to be required in order to use the 14926 receptor in the utilities asserted by the Applicant.

Under the Guidelines, "[t]he examiner's decision [with respect to patentable utility] must be supported by a preponderance of all the evidence of record," MPEP § 2107.02, *citing In re Oetiker*, 24 U.S.P.Q.2d 1443 (Fed. Cir. 1992). In the present case, Applicants' references constitute the only evidence of record demonstrating the view of those of skill in the art regarding the utility of GPCR sequences. Accordingly, the preponderance of the evidence supports Applicants' asserted utility.

III. The inventions of claims 32-59 have patentable utility.

In *Cross v. Iizuka* (753 F.2d 1040 (Fed. Cir. 1985)), the court held that "[w]hen a properly claimed invention meets at least one stated objective, utility under §101 is clearly shown." *Cross*, 753 F.2d at 1046 fn9, citing *Raytheon Co. v. Roper Corp.* 724 F.2d 951, 958 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 835 (1984). Thus, in order to reject the claims for the lack of patentable utility, the Examiner must demonstrate the invalidity of each of Applicants' asserted uses. In the present case, the Applicants have demonstrated that the 14926 receptor has specific, substantial, and credible utility in selectivity screening and drug screening for therapeutics that target GPCRs. The Examiner has maintained the rejection of the claims under 35 U.S.C. § 101, but has not provided evidence or factual findings to demonstrate that one of skill in the art would not find the utility asserted by the Applicant to be credible, or that additional research would be required to use the 14926 receptor as described by the Applicants. Accordingly, all of the evidence of record supports Applicants' asserted utility. For these reasons, the rejection of the claims under 35 U.S.C. §101 should be reversed.

Issue 2—Whether the invention of claims 52-59 is enabled under 35 U.S.C. § 112, first paragraph.

The Examiner has maintained the rejection of claims 52-59 under 35 U.S.C. § 112, first paragraph, on the grounds that these claims recite a step of determining whether a test compound modulates the activity of a 14926 polypeptide but have not disclosed which activity to measure. However, the specification provides guidance regarding G-protein mediated signaling pathways, including pathways mediated by phophatidylinositol turnover and pathways mediated by cyclic AMP turnover and metabolism. *See*, line 28 of page 7 through line 23 of page 9 of the specification. Furthermore, methods for assaying these signal transduction pathways are well known in the art. *See*, for example, Kenakin (1996) *Pharmacol. Rev.* 48:413-63; and Filtz *et al.* (1994) *Mol. Pharmacol.* 46:8-14, provided herewith as **Appendix D** and **Appendix E**. Accordingly, based on the guidance provided in the specification, one of skill in the art would be

able to determine whether a test compound modulates the activity of a 14926 polypeptide as recited in the claim.

For these reasons, the rejection of claims 52-59 under 35 U.S.C. §112, first paragraph, for lack of enablement should be reversed.

Issue 3—Whether the invention of claims 37-46 and 54-57 meets the written description requirement set forth in 35 U.S.C. § 112, first paragraph.

The Examiner has maintained the rejection of claims 37-46 and 54-57 under 35 U.S.C. § 112, first paragraph, on the grounds that the specification does not provide a sufficient written description of the polypeptides used in the methods of these claims.

Applicants have presented arguments demonstrating that the sequence variants recited in claims 37-46 and 54-57 are described by both their structural properties and their functional properties and therefore these polypeptides are adequately described. However, the Examiner states that in order to provide sufficient written description of the claimed sequence variants, the specification must disclose "which mutation or substitutions would be tolerated for keeping an activity." December 14, 2001 Office Action, page 7. Thus the Examiner appears to require that the specification disclose the sequence of each variant falling within the structural and functional limitations set forth in the claims in order to adequately describe the claimed genus of sequences. However, this requirement is not supported by the "Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, ¶ 1, 'Written Description' Requirement" (66 Fed. Reg. 1099 (2001)) and the supporting case law.

The "Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, ¶ 1, 'Written Description' Requirement" state that genus may be described by "sufficient description of a representative number of species . . . or by disclosure of relevant, identifying characteristics , *i.e.* structure or other physical and/or chemical properties." *Id.* at 1106. Furthermore, the Guidelines state that "[d]isclosure of any combination of . . . identifying characteristics that distinguish the claimed invention from other materials and would lead one to the conclusion that

the applicant was in possession" of the claimed invention is sufficient to satisfy the written description requirement. *Id.* at 1106.

Applicants submit that the written description provided for the polypeptides recited in claims 37-46 and 54-57 meet this requirement. The claims recite the identifying structural characteristics that define each genus of nucleotide sequences or amino acid sequences. Claims 37 and 54 recite polypeptides comprising an amino acid sequence having at least 70, 80%, or 90% sequence identity with amino acid sequence shown in SEQ ID NO:2. Claims 42 and 56 recite polypeptides comprising the amino acid sequence of a sequence variant of the amino acid sequence shown in SEQ ID NO:1, where the sequence variant is encoded by a nucleotide sequence that hybridizes to the nucleotide sequence shown in SEQ ID NO:2 under the specified stringent conditions. The structural limitations in these claims are sufficient to distinguish the claimed nucleotide sequences and amino acid sequences from other materials and thus sufficiently define the claimed genus.

Furthermore, in *Regents of the University of California v. Eli Lilly & Co*, 119 F.3d 1559, 1569 (Fed. Cir. 1997), the court held that "[a] description of a genus of cDNAs may be achieved by means of a recitation of a representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus or of a recitation of structural features common to the members of the genus, which features constitute a substantial portion of the genus." The recitation of the structural features of sequence identity with SEQ ID NO:1 or hybridization under stringent conditions with SEQ ID NO:2 is sufficient to satisfy this requirement.

Applicant has further provided the functional characteristics that distinguish the claimed sequences of the genus. Claims 37, 42, 54, 56, and their dependent claims are drawn to a genus of polypeptides having G-protein mediated signal transduction activity. Accordingly, each genus recited in claims 37-46 and 54-57 has been described by both its structural and functional features.

Example 14 of the " Revised Interim Written Description Guidelines Training Materials " (www.uspto.gov/web/menu/written.pdf) demonstrates that when the structural and functional features of the sequences encompassed by a genus are described, the description of the genus

meets the requirements of 35 U.S.C. § 112, first paragraph. Example 14 is directed to a genus of proteins having at least 95% sequence identity to the sequence of SEQ ID NO:3, wherein the proteins in the genus catalyze the reaction A → B. The conclusion in the analysis of this example is that the generic claim of Example 14 is sufficiently described under § 112, first paragraph, because (1) "the single sequence disclosed in SEQ ID NO:3 is representative of the genus" and (2) the claim recites a limitation requiring the compound to catalyze the reaction from A → B. The conclusion in the Training Materials is that one of skill in art would recognize that the applicants were in possession of the necessary common attributes possessed by the members of the genus.

Following the analysis of Example 14, Applicant submits that claims 37-46 and 54-57 satisfy the written description requirements of § 112, first paragraph. Specifically, the claims of the present invention encompass methods of using amino acid sequences having sequence identity to the amino acid sequence set forth in SEQ ID NO:2 or amino acid sequence encoded by a nucleotide sequence that hybridizes under specified conditions to the nucleotide sequence set forth in SEQ ID NO:8. As in Example 14, the specification discloses the nucleic acid sequence (SEQ ID NO:1) and encoded amino acid sequence (SEQ ID NO:2) and the claims recite a limitation requiring the recited polypeptides to have a specific function (*i.e.* G-protein mediated signal transduction activity). Accordingly, claims 37-46 and 54-57 provide the relevant, identifying characteristics that describe the claimed genus, and one of skill in the art would recognize that the inventors were in possession of the claimed invention.

For these reasons, the rejection of claims 37-46 and 54-57 under 35 U.S.C. §112, first paragraph, for lack of written description should be reversed.

CONCLUSION

In view of the arguments presented above, Applicant contends that each of claims 32-59 is patentable. Therefore, reversal of the rejections under 35 U.S.C. §101 and 35 U.S.C. §112, first paragraph, is respectfully solicited.

Respectfully submitted,

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In re: Glucksmann et al.
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APPENDIX A

In re:	Glucksmann *et al.*	Confirmation No.	6988
Appl. No.:	09/383,745	Group Art Unit:	1646
Filed:	August 26, 1999	Examiner:	E. Lazar Wesley
For:	14926 RECEPTOR, A NOVEL G-PROTEIN COUPLED RECEPTOR		

APPEALED CLAIMS

32. (Amended) A method for modulating the activity of a polypeptide comprising the amino acid sequence shown in SEQ ID NO:1; the method comprising contacting the polypeptide with a compound under conditions that allow the compound to modulate the activity of the polypeptide, wherein the activity of the polypeptide is modulated in a cell selected from the group consisting of brain cells, spleen cells, lung cells, kidney cells, skeletal muscle cells, liver cells, and heart cells.

33. (New) The method of claim 32, wherein said compound is an antibody.

34. (New) The method of claim 32, wherein said cell is a brain cell.

35. (New) The method of claim 32 wherein the activity of the polypeptide is modulated in a subject having a disorder associated with hyperplasia or inflammation.

36. (New) The method of claim 32, wherein said activity is a G-protein mediated signal transduction activity.

37. (Amended) A method for modulating the activity of a polypeptide comprising an amino acid selected from the group consisting of:

(a) the amino acid sequence of a sequence variant of the amino acid sequence shown in SEQ ID NO:1, wherein said sequence variant has G-protein mediated signal transduction activity and has at least about 70% sequence identity with the amino acid sequence shown in SEQ ID NO:1;

(b) the amino acid sequence of a sequence variant of the amino acid sequence shown in SEQ ID NO:1, wherein said sequence variant has G-protein mediated signal transduction activity and has at least about 80% sequence identity with the amino acid sequence shown in SEQ ID NO:1;

(c) the amino acid sequence of a sequence variant of the amino acid sequence shown in SEQ ID NO:1, wherein said sequence variant has G-protein mediated signal transduction activity and has at least about 90% sequence identity with the amino acid sequence shown in SEQ ID NO:1;

the method comprising contacting the polypeptide with a compound under conditions that allow the compound to modulate the activity of the polypeptide, wherein the activity of the polypeptide is modulated in a cell selected from the group consisting of brain cells, spleen cells, lung cells, kidney cells, skeletal muscle cells, liver cells, and heart cells.

38. (New) The method of claim 37, wherein said compound is an antibody.

39. (New) The method of claim 37, wherein said cell is a brain cell.

40. (New) The method of claim 37 wherein the activity of the polypeptide is modulated in a subject having a disorder associated with hyperplasia or inflammation.

41. (New) The method of claim 37, wherein said activity is a G-protein mediated signal transduction activity.

42. (Amended) A method for modulating the activity of a polypeptide comprising the amino acid sequence of a sequence variant of the amino acid sequence shown in SEQ ID NO:1, wherein said sequence variant has G-protein mediated signal transduction activity and is encoded by a nucleotide sequence that hybridizes to the nucleotide sequence shown in SEQ ID NO:2 under stringent conditions comprising hybridization in 6X SSC at about 45°C followed by one or more washes in 0.2X SSC/0.1%SDS at 50-65°C; said method comprising contacting the polypeptide with a compound under conditions that allow the compound to modulate the activity of the

polypeptide, wherein said modulation is in a cell selected from the group consisting of brain cells, spleen cells, lung cells, kidney cells, skeletal muscle cells, liver cells, and heart cells.

43. (New) The method of claim 42, wherein said compound is an antibody.

44. (New) The method of claim 42, wherein said cell is a brain cell.

45. (New) The method of claim 42 wherein the activity of the polypeptide is modulated in a subject having a disorder associated with hyperplasia or inflammation.

46. (New) The method of claim 42, wherein said activity is a G-protein mediated signal transduction activity.

47. (Amended) A method for modulating the activity of a polypeptide comprising the amino acid sequence set forth as amino acids 6 to 370 of SEQ ID NO:1; said method comprising contacting the polypeptide with a compound under conditions that allow the compound to modulate the activity of the polypeptide, wherein said modulation is in a cell selected from the group consisting of brain cells, spleen cells, lung cells, kidney cells, skeletal muscle cells, liver cells, and heart cells.

48. (New) The method of claim 47, wherein said compound is an antibody.

49. (New) The method of claim 47, wherein said cell is a brain cell.

50. (New) The method of claim 47 wherein the activity of the polypeptide is modulated in a subject having a disorder associated with hyperplasia or inflammation.

51. (New) The method of claim 47, wherein said activity is a G-protein mediated signal transduction activity.

52. (New) A method for identifying a compound that modulates the activity of a polypeptide comprising the amino acid sequence shown in SEQ ID NO:1; the method comprising contacting a cell expressing the polypeptide with a test compound under conditions such that the test compound can modulate the activity of the polypeptide and assessing the activity of the polypeptide to thereby determine if the test compound is a compound that modulates the activity of the polypeptide, wherein the cell is selected from the group consisting of brain cells, spleen cells, lung cells, kidney cells, skeletal muscle cells, liver cells, and heart cells.

53. (New) The method of claim 52 wherein the activity of the polypeptide that is modulated is G-protein-mediated signal transduction activity.

54. (New) A method for identifying a compound that modulates the activity of a polypeptide comprising an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of a sequence variant of the amino acid sequence shown in SEQ ID NO:1, wherein said sequence variant has G-protein mediated signal transduction activity and has at least about 70% sequence identity with the amino acid sequence shown in SEQ ID NO:1;

(b) the amino acid sequence of a sequence variant of the amino acid sequence shown in SEQ ID NO:1, wherein said sequence variant has G-protein mediated signal transduction activity and has at least about 80% sequence identity with the amino acid sequence shown in SEQ ID NO:1;

(c) the amino acid sequence of a sequence variant of the amino acid sequence shown in SEQ ID NO:1, wherein said sequence variant has G-protein mediated signal transduction activity and has at least about 90% sequence identity with the amino acid sequence shown in SEQ ID NO:1;

the method comprising contacting a cell expressing the polypeptide with a test compound under conditions such that the test compound can modulate the activity of the polypeptide and assessing the activity of the polypeptide to thereby determine if the test compound is a compound that modulates the activity of the polypeptide, wherein the cell is selected

from the group consisting of brain cells, spleen cells, lung cells, kidney cells, skeletal muscle cells, liver cells, and heart cells.

55. (New) The method of claim 54 wherein the activity of the polypeptide that is modulated is G-protein-mediated signal transduction activity.

56. (New) A method for identifying a compound that modulates the activity of a polypeptide comprising the amino acid sequence of a sequence variant of the amino acid sequence shown in SEQ ID NO:1, wherein said sequence variant has G-protein mediated signal transduction activity and is encoded by a nucleotide sequence that hybridizes to the nucleotide sequence shown in SEQ ID NO:2 under stringent conditions comprising hybridization in 6X SSC at about 45°C followed by one or more washes in 0.2X SSC/0.1%SDS at 50-65°Cthe method comprising contacting a cell expressing the polypeptide with a test compound under conditions such that the test compound can modulate the activity of the polypeptide and assessing the activity of the polypeptide to thereby determine if the test compound is a compound that modulates the activity of the polypeptide, wherein the cell is selected from the group consisting of brain cells, spleen cells, lung cells, kidney cells, skeletal muscle cells, liver cells, and heart cells.

57. (New) The method of claim 56 wherein the activity of the polypeptide that is modulated is G-protein-mediated signal transduction activity.

58. (New) A method for identifying a compound that modulates the activity of a polypeptide comprising the amino acid sequence set forth as amino acids 6 to 370 of SEQ ID NO:1, the method comprising contacting a cell expressing the polypeptide with a test compound under conditions such that the test compound can modulate the activity of the polypeptide and assessing the activity of the polypeptide to thereby determine if the test compound is a compound that modulates the activity of the polypeptide, wherein the cell is selected from the group consisting of brain cells, spleen cells, lung cells, kidney cells, skeletal muscle cells, liver cells, and heart cells.

59. (New) The method of claim 58 wherein the activity of the polypeptide that is modulated is G-protein-mediated signal transduction activity.

In re: Glucksmann et al.
Appl. No. 09/383,745
Filed August 26, 1999

APPENDIX B

Orphan G protein-coupled receptors: a neglected opportunity for pioneer drug discovery

Jeffrey M. Stadel, Shelagh Wilson and Derk J. Bergsma

Access to DNA databases has introduced an exciting new dimension to the way biomedical research is conducted. 'Genomic research' offers tremendous opportunity for accelerating the identification of the cause of disease at the molecular level and thereby foster the discovery of more selective medicines to improve human health and longevity. The current challenge is to close the gap rapidly between gene identification and clinical development of efficacious therapeutics. In the present review, **Jeffrey Stadel, Shelagh Wilson and Derk Bergsma** outline the rationale and describe strategies for converting one large class of novel genes, orphan G protein-coupled receptors (GPCRs), into therapeutic targets. Historically, the superfamily of GPCRs has proven to be among the most successful drug targets and consequently these newly isolated orphan receptors have great potential for pioneer drug discovery.

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The advent of rapid DNA sequencing spawned the 'genomic era', which has led to the initiation of the Human Genome Project. The novel technologies developed in association with genomic research have already had a significant impact on the way investigations into the basis of disease are being conducted and will, no doubt, substantially enhance the means by which diseases are diagnosed and treated in the near future. To keep pace with the evolution of molecular medicine, the pharmaceutical industry has embraced genomics and is attempting to exploit the new technologies to identify novel targets for drug discovery. The major questions that remain to be addressed concern how to convert genomic sequences into therapeutic targets in an expeditious manner and eventually to obtain pharmaceutical drugs that will enhance the quality of life. This review will deal with a single class of novel molecular targets, focusing on the burgeoning collection of G protein-coupled receptors (GPCRs) called 'orphan' receptors[1]. GPCRs are a superfamily of integral plasma membrane proteins involved in a broad array of signalling pathways. Since the first cloning of GPCR gene sequences over a decade ago, novel members of the GPCR superfamily have continued to emerge through cloning activities as well as through bioinformatic analyses of sequence databases, although their ligands are unidentified and their physiological relevance remain to be defined. These 'orphan' receptors provide a rich source of potential targets for drug discovery.

The members of the GPCR superfamily are related both structurally and functionally. The signature motif of these receptors is seven distinct hydrophobic domains, each of which is 20–30 amino acids long and which are linked by hydrophilic amino acid sequences of varied length[2,3]. Biophysical[4] and biochemical[5] studies support the notion that these receptors are intercalated into the plasma membrane with the amino terminus extracellular and the carboxy terminus in the cytoplasmic portion of the cell. Therefore, these receptors are often referred to as seven transmembrane (or 7TM) receptors. While it is not yet known how many individual genes actually encode these receptors, it is clear that this family of proteins is one of the largest yet identified. Functionally, GPCRs share in common the property that upon agonist binding they transmit signals across the plasma membrane through an interaction with heterotrimeric G proteins[6,7]. These receptors respond to a vast range of agents[2,5,8] such as protein hormones, chemokines, peptides, small biogenic amines, lipid-derived messengers, divalent cations (e.g. a Ca^{2+} sensor has been identified that is a GPCR)[9] and even proteases such as thrombin, which activates its receptor by cleaving off a portion of the amino terminus[10]. Finally, these receptors play an important role in sensory perception including vision and smell[2,5,8]. Correlated with the broad range of agents that activate these receptors is their existence in a wide variety of cells and tissue types, indicating that they play roles in a diverse range of physiological processes. It is likely, therefore, that the GPCR superfamily is involved in a variety of pathologies. This point was recently emphasized by the surprising discovery that certain GPCRs for chemokines act as co-factors for HIV infection[11–13].

GPCRs represent the primary mechanism by which cells sense alterations in their external environment and convey that information to the cells' interior. The binding of an agonist to the receptor promotes conformational changes in the cytoplasmic domains that lead to the interaction of the receptor with its cognate G protein(s). Agonist-promoted coupling between receptors and G proteins leads to the activation of intracellular effectors that substantially amplify the production of second messengers feeding into the signalling cascade. Since effectors are often enzymes [e.g. adenylate cyclase[14], which converts ATP to cAMP, or phospholipase C (Ref. 15), which hydrolyses inositol lipids in membranes to release inositol trisphosphate, which in turn mobilizes Ca^{2+} within a cell] or ion channels[16], many second messenger molecules can be produced as the result of a single agonist binding event with its receptor. Changes in the intracellular levels of ions or cAMP, or both,

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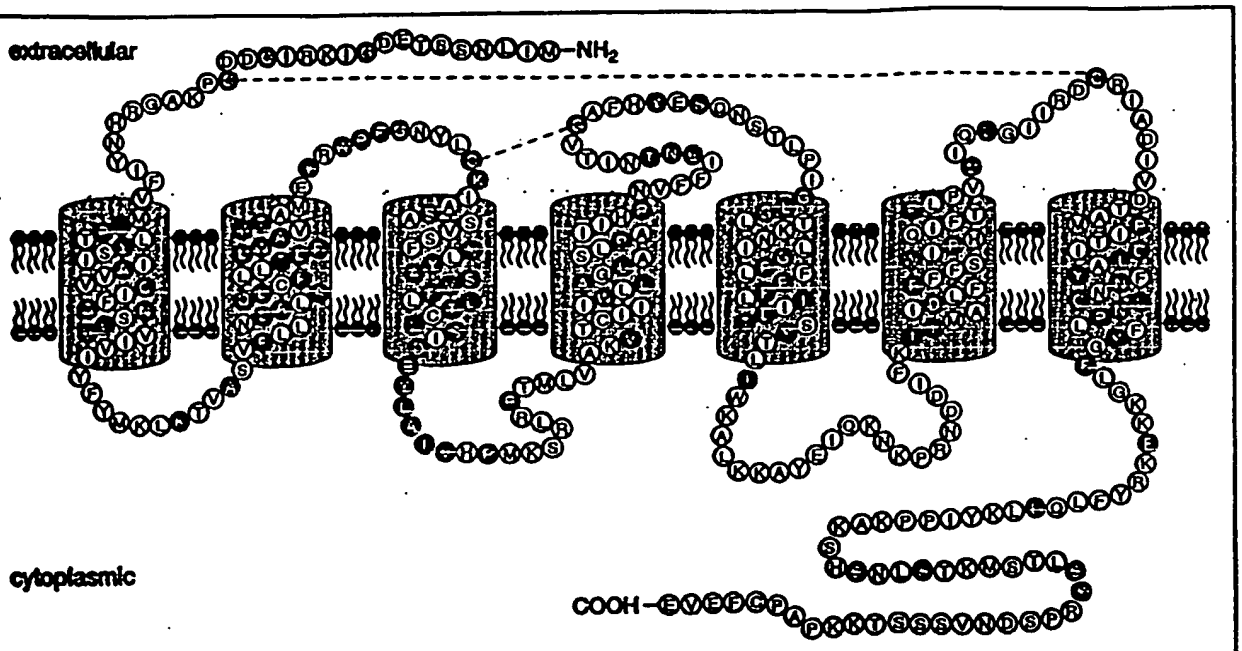

Fig. 1. Comparison of the protein sequence identity of the orphan APJ[19] receptor with the angiotensin AT_1 receptor[20]. The filled circles indicate amino acid identity (29.9%) between the two G protein-coupled receptors (GPCRs). This is a typical example of the protein sequence identity shared between orphan and known GPCRs.

result in the modulation of distinct phosphorylation cascades[17,18], extending through the cytosol to the nucleus, that eventually culminate in the physiological response of the cell to the extracellular stimulus. Although the overall paradigm is apparently the same for all GPCRs, the diversity of receptors, G proteins and effectors suggest a myriad of potential signalling processes and this becomes an important concept as we try to identify the function of orphan GPCRs.

To date, more than 800 GPCRs have actually been cloned from a variety of eukaryotic species, from fungi to humans [see L. F. Kolakowski in GCRDb-WWW The G Protein-Coupled Receptor DataBase World-Wide-Web Site (http://receptor.mgh.harvard.edu/GCRDBHOME.html.org)]. For humans, the most represented species, about 140 GPCRs have been cloned for which the cognate ligands are also known. This number excludes the sensory olfactory receptors, of which hundreds to thousands are predicted to exist. By traditional molecular genetic approaches, coupled with the explosion in genomic information, it has been possible to identify more than 100 additional orphan GPCR family members. By definition, there is enough sequence information in the receptor cDNAs to place them clearly in the superfamily of GPCRs, but often there is insufficient sequence homology with known members of this family to be able to assign their ligands with confidence or predict their function. In total, there are currently over 240 human GPCRs, excluding sensory receptors. As the size of sequence databases continues to increase, this list is expected to grow to 400, and perhaps even to 1000 or more unique gene products. The list will grow even further as paralogues and alternatively spliced GPCR variants emerge. Most orphan GPCRs share a low degree of sequence homology (typically about 25–35% overall amino acid sequence identity), with known GPCRs, suggesting that they belong to new subgroups of receptors (Fig. 1)[19,20]. Indeed, several orphan GPCRs show closer homology to each other than to known GPCRs. Nevertheless, the majority of orphan receptors are phylogenetically distributed among a broad spectrum of distantly related, known receptor subgroups.

What is the rationale for investing considerable time and resources into trying to establish the function of orphan GPCRs? Simply stated, GPCRs have a proven history of being excellent therapeutic targets. Within the past 20 years, several hundred new drugs have been registered that are directed towards activating or antagonizing GPCRs; in fact, it is estimated that most current research within the pharmaceutical industry is focused on this signalling pathway[21]. Table 1 shows a representative snapshot of a variety of receptors, disease targets and corresponding drugs. It is clear from this table that the therapeutic targets span a wide range of disorders and disease states. Another example of the significance and versatility of GPCRs is the number of cases of genetic diseases that are linked to defects in these proteins; some of these diseases are indicated in Table 2 (Refs 22–38). It is likely that many more genetic diseases will be mapped to GPCRs as the era of genomics continues to expand and families with inherited mutations are examined much more comprehensively.

The importance of GPCRs to drug discovery continues to be manifested by the fact that across the pharmaceutical industry active research projects, ranging from basic studies all the way through to advanced development, are focused on GPCRs as primary targets. Molecular biology has had a dramatic influence on these efforts.

Table 1. Examples of marketed drugs for G protein-coupled receptors (GPCRs)

GPCR	Generic	Drug	Indication
Muscarinic acetylcholine	Bethanechol	Urecholine	GI
	Dicyclomine	Bentyl	GI
	Ipratropium	Atrovent	CP
Adrenoceptor			
β_1	Atenolol	Tenormin	CP
α_2	Clonidine	Catapres	CP
β_1/β_2	Propranolol	Inderal	CP
α_1	Terazosin	Hytrin	CP
β_2	Albuterol	Ventolin	CP
$\beta_1/\beta_2/\alpha_1$	Carvedilol	Coreg	CP
Angiotensin			
AT_1	Losartan	Cozaar	CP
	Eprosartan	Teveten	CP
Calcitonin	Calcitonin	Calcimar	Osteoporosis
	eel-Calcitonin	Elcatonin	Osteoporosis
Dopamine			
D_2	Metoclopramide	Reglan	GI
D_2/D_3	Ropinirole	Requip	CNS
D_2	Haloperidol	Haldol	CNS
Gonadotropin-releasing factor	Goserelin	Zoladex	Cancer
	Nafarelin	Synarel	Endometriosis
Histamine			
H_1	Dimenhydrinate	Dramamine	CNS
H_1	Terfenadine	Seldane	CP
H_2	Cimetidine	Tagamet	GI
H_2	Ranitidine	Zantac	GI
Serotonin (5-HT)			
$5\text{-}HT_{1D}$	Sumatriptan	Imitrex	CNS
$5\text{-}HT_{2A}$	Ritanserin	Tisertan	CNS
$5\text{-}HT_4$	Cisapride	Propulsid	GI
$5\text{-}HT_{1B}$	Trazodone	Desyrel	CNS
$5\text{-}HT_{2A/2C}$	Clozapine	Clozaril	CNS
Leukotriene	Pranlukast	Onon	CP
	Zafirlukast	Accolate	CP
Opioid			
κ	Buprenorphine	Buprenex	CNS
	Butorphanol	Stadol	CNS
μ	Alfentanil	Alfenta	CNS
	Morphine	Kadian	CNS
Oxytocin		Syntocinon	Labour
Prostaglandin	Epoprostenol	Flolan	CP
	Misoprostol	Cytotec	GI
Somatostatin	Octreotide	Sandostatin	Cancer
Vasopressin	Desmopressin		CP/Renal

CP, cardiopulmonary system; GI, gastrointestinal system.

Table 2. Diseases associated with mutations of G protein-coupled receptors (GPCRs)

GPCR	Mutation	Disease	Refs
Rhodopsin	Missense: Pro23 to His (NT) Missense: Val87 to Asp (2TM) Missense: Tyr178 to Cys (2EL) Nonsense: Gln344 to Stop (CT)	Retinitis pigmentosa	22, 23
Thyroid stimulating hormone	Missense: Asp619 to Gly (3IL) Missense: Ala623 to Ile (3IL)	Hyperfunctioning thyroid adenomas	24
Luteinizing hormone	Missense: Asp578 to Gly (6TM)	Precocious puberty	25
Vasopressin V_2	Missense: Arg137 to His (2IL) Missense: Gly185 to Cyc (2EL) Frameshift at Arg230 (3TM)	X-linked nephrogenic diabetes	26–28
Ca^{2+}	Missense: Arg186 to Glu (NT) Missense: Glu298 to Lys (NT) Missense: Arg796 to Trp (3IL) Missense: Glu128 to Ala (NT)	Hyperparathyroidism, hypocalciuric hypercalcaemia	29, 30
Parathyroid hormone (PTH type b)	Missense: His223 to Arg (1IL)	Short-limbed dwarfism	31
β_3-Adrenoceptor	Missense: Trp64 to Arg (1IL)	Obesity, NIDDM	32–34
Growth-hormone-releasing hormone	Nonsense: Glu72 to Stop (NT)	Dwarfism	35
Adrenocorticotropin	Missense: Ser74 to Ile (2TM)	Glucocorticoid deficiency	36
Glucagon	Missense: Gly40 to Ser (NT)	Diabetes, hypertension	37, 38

Abbreviations: CT, carboxyl terminus; EL, extracellular loop; IL, intracellular loop; NIDDM, non-insulin-dependent diabetes mellitus; NT, amino terminus; TM, transmembrane segment.

The cloning of cDNAs for well-known GPCRs led to the discovery of a surprising number of paralogues[5]. The existence of these novel receptor subtypes was unexpected because the current cornucopia of pharmacological agents does not possess the required selectivity to distinguish all of them clearly, and thus an opportunity for drug discovery was quickly recognized. Current research efforts seek to define the physiology associated with these novel receptor subtypes and to discover highly selective compounds as potential pharmaceutical drugs. These efforts are almost exclusively focused on GPCRs for which activating ligands are known. Since characterized GPCRs were, and continue to be, attractive therapeutic targets, it is most reasonable to speculate that many of the orphan receptors have similar potential. The initial challenge is to determine the function of each orphan receptor through the identification of activating ligands and, once the function is clarified, link the orphan receptor to a specific disease and thus establish it as a candidate for a comprehensive drug discovery effort.

Reverse molecular pharmacology

Until recently, research into the identification of GPCRs as targets for drug discovery has been conducted using the traditional approach illustrated in Fig. 2. For this strategy, the starting point is functional activity, which forms the basis of an assay by which a ligand is identified through purification from biological fluids, cell supernatants or tissue extracts. One example of the success of this strategy is the discovery of the potent vasoconstricting peptide endothelin[39]. Once isolated, the ligand is used to characterize its cellular and tissue biology as well as its pathophysiological role. Subsequently, cDNAs encoding corresponding receptors are 'fished' from gene libraries using a variety of methodologies (e.g. receptor purification and expression cloning) that often either directly or indirectly use the ligand as the 'hook'. As the nucleotide sequences for GPCRs begin to accumulate and be analysed, additional receptors can be cloned by homology screening, by positional cloning, and by polymerase chain reaction (PCR) methodologies that use oligonucleotide primers based on nucleotide sequences conserved within the seven transmembrane domains of the GPCR family. Once the cloned human receptor cDNA is expressed in a heterologous cell system[40], it is used, together with its ligand, to form the basis of a screen to explore chemical compound libraries for receptor antagonists or agonists. Lead structures identified in the screen are refined through medicinal chemistry using an iterative process. Resulting drug leads with appropriate *in vivo* pharmacology are passed on into the clinic for development.

Recently, this paradigm has changed radically with the introduction of a new reverse molecular pharmacological

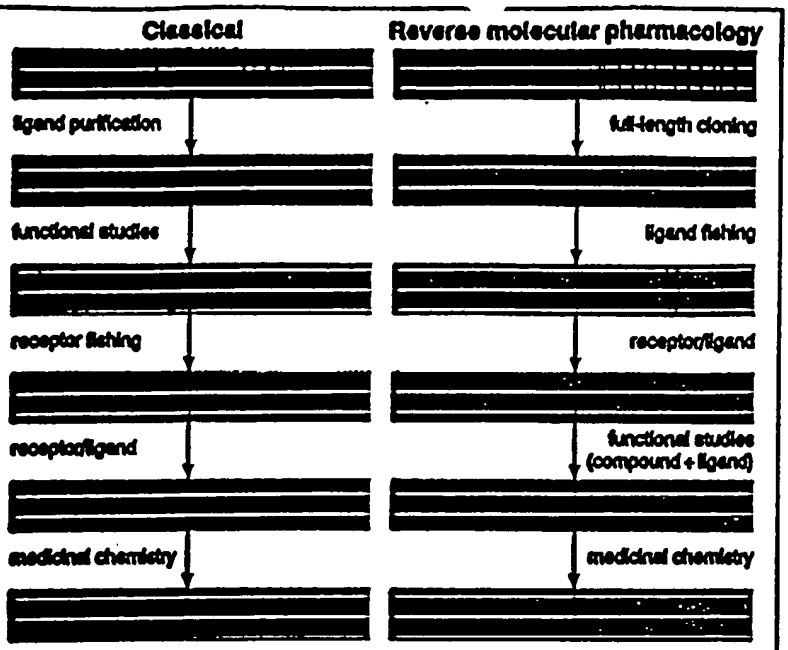

Fig. 2. Paradigm shift from classical to reverse molecular pharmacological approaches for drug discovery.

strategy, shown diagramatically in Fig. 2. Through both traditional molecular cloning techniques and, more recently, mass sequencing of expressed sequence tags (ESTs) from cDNA libraries, it is now possible to identify GPCRs through computational or bioinformatic methodologies. The EST approach, initially proposed by Sidney Brenner (University of Cambridge) and first brought to large-scale practice by Craig Venter (The Institute of Genome Research), constitutes random, single-pass sequencing of cDNAs randomly picked from a collection of cDNA libraries, followed by extensive bioinformatic analysis of the sequence to identify structural signatures characteristic of GPCRs. Once new members of the GPCR superfamily are identified, the recombinantly expressed receptors are used in functional assays to search for the associated novel ligands. The receptor–ligand pair are then used for compound bank screening to identify a lead compound that, together with the activating ligand, is used for biological and pathophysiological studies to determine the function and potential therapeutic value of a receptor antagonist (or agonist) in ameliorating a disease process. In addition, clues as to therapeutic potential may involve receptor genotyping of disease populations. Once a link with a disease is finally identified, an appropriate compound can be advanced for clinical study.

The reverse molecular pharmacological strategy is a far more daunting challenge and risky endeavour when compared with the more traditional approach, since the starting material for a drug discovery effort is simply an orphan receptor of unknown function, with no apparent relationship to a disease indication. However, the potential reward of using this approach is that resultant drugs naturally will be pioneer or innovative discoveries, and a significant proportion of these unique drugs may be useful to treat diseases for which existing therapies are lacking or insufficient.

Screening strategy

Figure 3 illustrates the generic strategy that we use for our reverse molecular pharmacological approach. In addition to the EST approach, which has yielded the majority of our collection of orphan receptors, we have also used a number of more traditional approaches such as low-stringency screening, using portions of known GPCRs as hybridization probes, as well as PCR-based methods. By these techniques we have succeeded in identifying more than 70 orphan receptors in addition to those already cited in the literature.

Since cDNAs identified by EST cloning are often incomplete, northern hybridization analysis is used to establish the tissue or cell pattern of mRNA expression of the GPCRs. This information is used to identify the tissue or cell cDNA libraries that are to be probed for full-length clones and, significantly, to determine whether a receptor is expressed in a particular normal or diseased tissue of interest. A highly selective tissue expression pattern may also provide a clue with respect to receptor function. Once obtained, full-length GPCR clones are expressed in mammalian cell lines and yeast model systems (see below) for functional analysis. *Xenopus* oocytes may also be used for expression; however, low screening throughput limits their use to a secondary, confirmatory assay system. For mammalian cell expression, the human embryonic kidney (HEK) 293 cell line or Chinese hamster ovary (CHO) cells are frequently used. These cell types possess a large repertoire of G proteins that are necessary for coupling to downstream effectors *in situ*. They also share a reliable history of positive functional coupling for a wide variety of known GPCRs. However, since receptor coupling cannot be accurately predicted from primary sequence data, orphan GPCRs may need to be expressed in a variety of cell lines to establish viable coupling.

These heterologous expression systems form the basis for screening for an activating ligand. The success of establishing functional coupling of the recombinant receptor depends to a large extent on whether the receptor is properly expressed, which may be assessed by northern or Western blot analysis, and whether appropriate G proteins and downstream effectors are present in the cell in which the receptor is expressed. There are several major technical challenges to be met in order to initiate ligand fishing. Because it is difficult to predict accurately the coupling specificity of orphan GPCRs from their primary sequence, assays must be chosen that will detect a wide range of coupling mechanisms. These generally focus on changes in intracellular levels of cAMP or Ca^{2+} but can also include more generic measurements, such as metabolic activation of the cell via the cytosensor microphysiometer[41]. Recently, it has become possible to implement most of these screens in high-throughput format by using fluorescent-based

assays and using charge-coupled device cameras and reporter gene constructs that allow easy readout of the assay on microtitre plates. Ever increasing throughput of the assays will be necessary to screen large libraries. However, this approach is somewhat cumbersome and inefficient if all the assays described above have to be used. Is it possible to funnel heterologous signal transduction through a defined pathway? The prospect of an assay for a single transduction pathway comes from the observation that heterologous expression of the G protein subunit $G_{\alpha 15/16}$ promoted coupling of various GPCR subfamily members through activation of phospholipase Cβ and likely Ca^{2+} mobilization[42,43]. Although this approach may not work universally, the diversity of the GPCRs successfully coupled through $G_{\alpha 16}$ to phospholipid metabolism suggests that this could be a useful method to screen for orphan receptor activation.

Once heterologous receptor expression is achieved and functional assays are in place, ligand fishing experiments can be initiated. Although the homology with known GPCRs is low, we nevertheless begin by screening the orphans against known GPCR ligands; since the sequence homology between some subtypes of known receptors can be low (e.g. 30–40% between neuropeptide Y receptor subtypes), it is possible that new paralogue receptors for known ligands still remain to be discovered. The next step is to search for novel activating ligands by screening biological extracts obtained from tissues, biological fluids and cell supernatants. An additional option is screening libraries of compounds for activating ligands. Complex libraries of peptides or compound collections could be rich sources of 'surrogate' agonists that would promote receptor activation and coupling but are not endogenous ligands. The rationale for searching for surrogate agonists springs from a report that a nonpeptide agonist has been discovered for the angiotensin II receptor[44]. There is also an obvious precedent for nonpeptide agonists for opioid receptors. Screening of the very large libraries that will be generated by fractionation of biological extracts and by combinatorial chemical synthesis requires that the functional assays used have not only a high throughput but are also robust, since false positives can be a significant problem.

Examples are beginning to emerge from several efforts showing that progress has been made in characterizing orphan GPCRs. A first example is the identification of an orphan GPCR that functions as a calcitonin gene-related peptide (CGRP) receptor[45]. CGRP is a peptide of 37 amino acids, widely distributed in neurones, and functions as a potent vasodilator. It may be involved in migraine and has been implicated in non-insulin-dependent diabetes mellitus because it promotes resistance to insulin. An orphan GPCR EST was derived from a human synovium cDNA library[45]. Sequence analysis showed that the new GPCR has ~56% similarity to the human calcitonin receptor and was hence originally expected to be a new subtype of the calcitonin receptor. The message for this novel receptor was expressed predominantly in lung, which is known to be a relatively rich source of CGRP receptors. Following full-length cloning from a human lung library, the orphan receptor cDNA was stably expressed in HEK293 cells. Both radioligand binding using ^{125}[I]CGRP, as well as functional assays of CGRP-stimulated cAMP accumulation, demonstrated an appropriate pharmacological profile for the expressed receptor similar to that observed with endogenous CGRP receptors on human neuroblastoma cells. In addition to identifying the CGRP receptor, the reverse molecular pharmacology approach has also been used to identify other orphan receptors, such as the anaphylatoxin C3a receptor[46].

The examples given above are for receptors with significant homology to known GPCR superfamily members and their activating ligands proved to be known GPCR ligands. Will ligand fishing be successful in identifying novel endogenous ligands? Recently, two groups

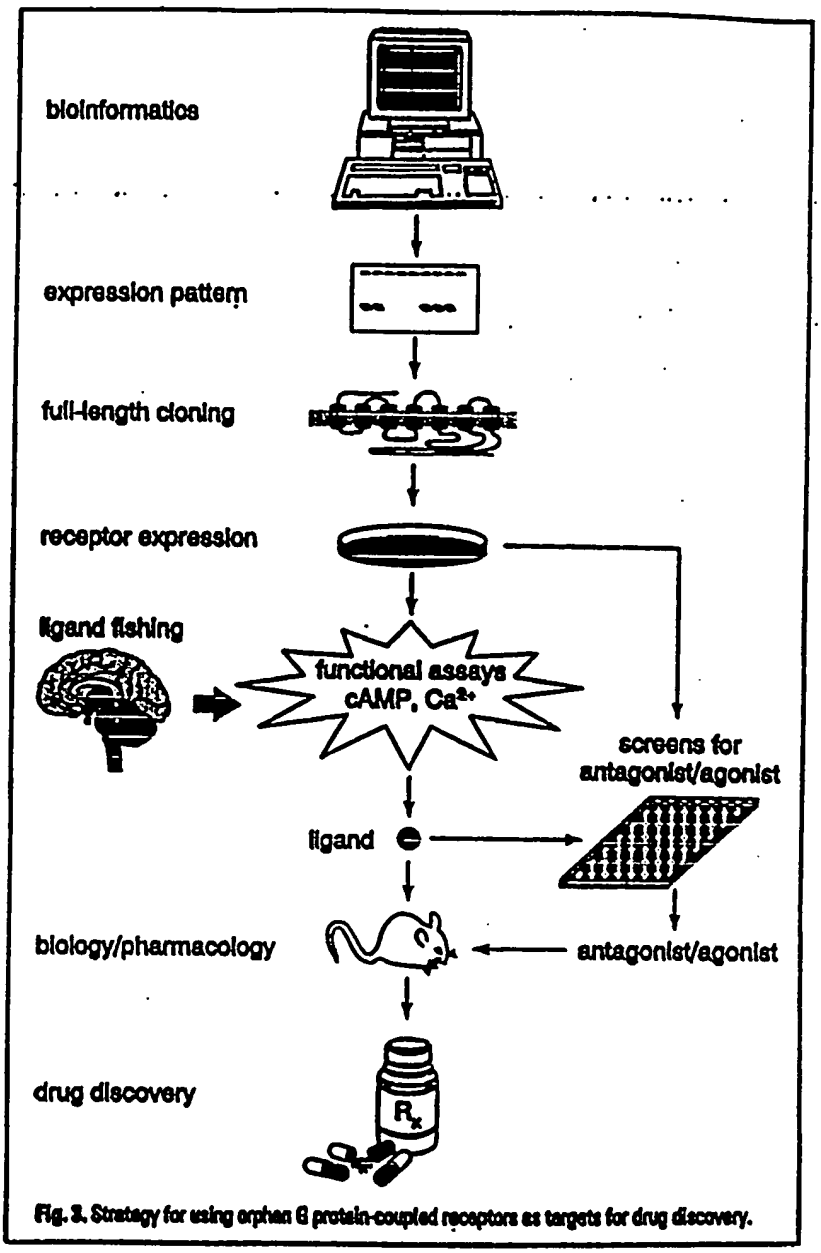

Fig. 3. Strategy for using orphan G protein-coupled receptors as targets for drug discovery.

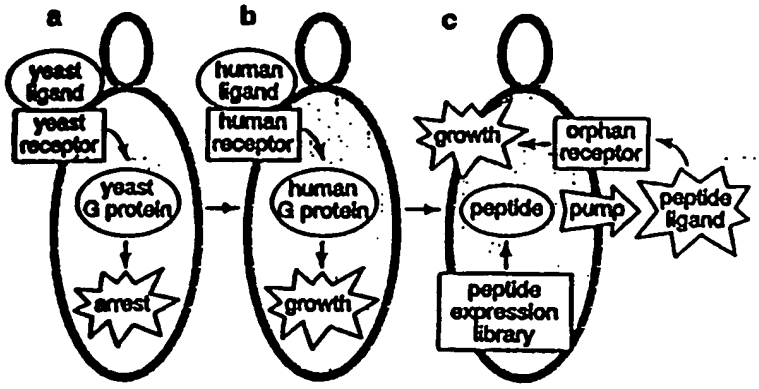

Fig. 4. Yeast-based screen for the identification of agonists for orphan G protein-coupled receptors (GPCRs). a: Normal, endogenous GPCR signalling in yeast (*Saccharomyces cerevisiae*). b: Substitution of a human GPCR and a human G_α subunit for yeast counterparts and modification of downstream signalling pathways such that agonist stimulation of the recombinant GPCR promotes growth. This yeast strain can be screened using biological extracts or compound libraries, or both. c: Yeast cells can be engineered to secrete small peptides from a random peptide library to identify autocrine surrogate, peptide agonists for recombinant orphan GPCRs. Modified from Ref. 49, with permission.

investigated an orphan opioid-like receptor, ORL1 (Refs 47 and 48). Both groups expressed the orphan GPCR in CHO cells and challenged the transfected cells with a series of opiate agonists, but without response. Both groups then used a similar ligand fishing approach. Taking crude extracts from rat brain[47] or porcine brain[48], they screened against the stably transfected cell lines using inhibition of adenylate cyclase activity as a functional assay. They were able to fractionate the brain extracts and identify the novel dynorphin-like ligand, which they called nociceptin[47] or orphanin FQ (Ref. 48). Thus, both teams successfully established a functional assay in transfected CHO cells that allowed the purification of a novel neuropeptide ligand that is 17 amino acids long for the orphan receptor. This work validates the ligand fishing approach for characterizing the function of orphan GPCRs.

Concluding remarks and future challenges

Although orphan GPCRs have been around for over ten years, very few companies have, until recently, been willing to risk their resources to explore opportunities among this category of receptors. However, the environment for the pharmaceutical industry has changed due to the confluence of several major technological advances. The conversion of gene sequences encoding GPCRs to drug targets is substantially aided by the development of combinatorial chemistry methods and miniaturized high-throughput screening techniques. The future challenge for drug discovery in this arena is to integrate these technologies innovatively and productively. One glimpse of the future comes from the field of functional genomics. The endogenous GPCR transduction system of the yeast, *Saccharomyces cerevisiae*, which is the pheromone pathway required for conjugation and mating, has been commandeered – through genetic engineering – to permit functional expression and coupling of human GPCRs and humanized G pr. .n subunits to the endogenous signalling machinery[49-51] (Fig. 4). Further manipulations involve conversion of the normal yeast response to pheromone or activating ligand (growth arrest) to positive growth on selective media or to reporter gene expression. In addition, yeast cells have been engineered to express and secrete small peptides from a random peptide library that will permit the autocrine activation of heterologously expressed human GPCRs (Refs 49 and 51). This provides an elegant means of screening rapidly for surrogate peptide agonists that activate orphan receptors. This yeast system is, of course, not limited to autocrine ligand screening but can also be used in high-throughput mode to screen directly the fractions from biological extracts and the various chemical libraries as described above. A major advantage of the yeast system over the mammalian heterologous expression systems is its ease of use and its lack of endogenous GPCRs, which can confound ligand fishing expeditions in mammalian cells.

There is now tremendous pressure to be the first on the market with highly selective drugs that target therapeutic areas of unmet medical need and ideally have novel mechanisms of action. As a consequence, the pharmaceutical industry has recognized the power of genomics to provide it with new and unique drug targets. Genomics has responded with a plethora of novel proteins, included among them over 100 orphan GPCRs. Because of the proven link of GPCRs to a wide variety of diseases and the historical success of drugs that target GPCRs, we believe that these orphan receptors are among the best targets of the genomic era to advance into the drug discovery process.

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CA_1A_2X-competitive inhibitors of farnesyltransferase as anti-cancer agents

Charles A. Omer and Nancy E. Kohl

For Ras oncoproteins to transform mammalian cells, they must be post-translationally farnesylated in a reaction catalysed by the enzyme farnesyl-protein transferase (FPTase). Inhibitors of FPTase have therefore been proposed as anti-cancer agents. In this review Charles Omer and Nancy Kohl discuss the development of FPTase inhibitors that are kinetically competitive with the protein substrate in the farnesylation reaction. These compounds are potent and selective inhibitors of the enzyme that block the tumourigenic phenotypes of *ras*-transformed cells and human tumour cells in cell culture and in animal models.

Since the identification of farnesyl-protein transferase (FPTase) activity in mammalian cells, there has been an intense effort to develop inhibitors of this housekeeping enzyme for use as potential, novel anti-cancer agents[1,2]. This idea stems from the fact that several of the proteins that regulate mammalian cell proliferation require a post-translational modification catalysed by this enzyme for biological activity. Efforts over the past eight years have yielded potent, cell-active inhibitors of FPTase that demonstrate anti-proliferative activity in cell culture and in rodent models of cancer.

The focus of the FPTase inhibitor (FTI) studies has been inhibition of the transforming activity of the Ras oncoproteins. Three *ras* genes, Ha-, N- and Ki-*ras*, encode four highly homologous, 21 kD proteins, Ha-, N-, Ki4A- and Ki4B-Ras (Ki4A- and Ki4B-Ras are encoded by splice variants of the Ki-*ras* gene)[3]. Ras functions to regulate the transduction of extracellular growth-promoting signals from membrane-bound receptor tyrosine kinases to intracellular growth-regulatory pathways. Typical of the low-molecular-weight G proteins, Ras is active when bound to GTP and inactive when bound to GDP. Cycling from the active to the inactive form is accomplished by the intrinsic GTPase activity of the protein. Mutations in Ras that abolish the GTPase activity result in constitutively active forms of the protein. Such oncogenically mutated forms of Ras, particularly Ki4B-Ras, are found in approximately 30% of many human cancers including 90% of pancreatic cancers and 50% of colon cancers[4,5].

Ras is synthesized as a biologically inactive, cytosolic protein that localizes to the inner surface of the plasma membrane where it acquires biological activity following a series of post-translational modifications (see Ref. 6 for review). The first and obligatory step in this series is the transfer of a 15-carbon isoprenoid, farnesyl, from farnesyl diphosphate (FPP) to the sulphur atom of the cysteine residue located four amino acids from the carboxyl terminus of the protein. This cysteine residue is part of the CA_1A_2X motif found in all FPTase protein substrates, where C is cysteine, A_1 and A_2 are usually aliphatic amino acids and X is usually serine, methionine, glutamine, alanine or cysteine. Following farnesylation, A_1A_2X is proteolytically cleaved and the now C-terminal farnesylcysteine is methylated. In the case of all of the Ras proteins except Ki4B-Ras, palmitate groups are then added to cysteine residues upstream of the farnesylated cysteine. The demonstration that farnesylation is essential for the transforming ability of the Ras oncoproteins[7–10] has spurred the development of inhibitors of the enzyme that catalyses this reaction, FPTase, as anti-cancer agents.

FPTase is a ubiquitously expressed, cytosolic enzyme comprised of two subunits, a 45 kDa α subunit and a 48 kDa β subunit[6]. Cross-linking studies have shown

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APPENDIX C

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A Regulatory Cascade of the Nuclear Receptors FXR, SHP-1, and LRH-1 Represses Bile Acid Biosynthesis

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Summary

Bile acids repress the transcription of cytochrome P450 7A1 (CYP7A1), which catalyzes the rate-limiting step in bile acid biosynthesis. Although bile acids activate the farnesoid X receptor (FXR), the mechanism underlying bile acid–mediated repression of *CYP7A1* remained unclear. We have used a potent, nonsteroidal FXR ligand to show that FXR induces expression of small heterodimer partner 1 (SHP-1), an atypical member of the nuclear receptor family that lacks a DNA-binding domain. SHP-1 represses expression of *CYP7A1* by inhibiting the activity of liver receptor homolog 1 (LRH-1), an orphan nuclear receptor that is known to regulate *CYP7A1* expression positively. This bile acid–activated regulatory cascade provides a molecular basis for the coordinate suppression of *CYP7A1* and other genes involved in bile acid biosynthesis.

Introduction

Cholesterol is essential for a number of cellular functions, including membrane biogenesis and steroid hormone and bile acid biosynthesis. However, in excess, cholesterol can contribute to disease processes such as atherosclerosis and gallstone formation. Therefore, cholesterol biosynthesis and catabolism must be coordinately regulated. The metabolism of cholesterol to bile acids represents a major pathway for its elimination from the body, accounting for approximately half of daily excretion. Cytochrome P450 7A (CYP7A1) is a liver-specific enzyme that catalyzes the first and rate-limiting step in one of the two pathways for bile acid biosynthesis (Chiang, 1998; Russell and Setchell, 1992). The gene encoding CYP7A1 is regulated by a variety of small, lipophilic molecules, including steroid and thyroid hormones, cholesterol, and bile acids. Notably, *CYP7A1* expression is stimulated by cholesterol feeding and repressed by bile acids. Thus, *CYP7A1* is under both feedforward and feedback regulation.

CYP7A1 expression is regulated by several members of the nuclear receptor superfamily of ligand-activated transcription factors (Chiang, 1998; Gustafsson, 1999; Russell, 1999). Recently, two nuclear receptors, the liver X receptor α (LXRα; NR1H3) (Apfel et al., 1994; Willy et al., 1995) and farnesoid X receptor (FXR; NR1H4) (Forman et al., 1995; Seol et al., 1995), were implicated in the feedforward and feedback regulation of *CYP7A1*, respectively (Peet et al., 1998; Russell, 1999). Both LXRα and FXR are abundantly expressed in the liver and bind to their cognate hormone response elements as heterodimers with the 9-*cis* retinoic acid receptor RXR (Mangelsdorf and Evans, 1995). LXRα is activated by the cholesterol derivative 24,25(*S*)-epoxycholesterol and binds to a response element in the *CYP7A1* promoter (Lehmann et al., 1997). Mice lacking LXRα do not induce *CYP7A1* expression in response to cholesterol feeding (Peet et al., 1998). Moreover, these animals accumulate massive amounts of cholesterol in their livers when fed a high cholesterol diet. These data establish LXRα as the cholesterol sensor responsible for feedforward regulation of *CYP7A1* expression.

Bile acids stimulate the expression of genes involved in bile acid transport, such as the intestinal bile acid–binding protein (*I-BABP*), and repress *CYP7A1* and other genes encoding enzymes involved in bile acid biosynthesis, such as CYP8B1, which converts chenodeoxycholic acid (CDCA) to cholic acid, and CYP27, which catalyzes the first step in the alternative, "acidic" pathway for bile acid synthesis (Russell and Setchell, 1992; Javitt, 1994; Russell, 1999). Recently, FXR was shown to be a bile acid receptor (Wang et al., 1996; Makishima et al., 1999; Parks et al., 1999). Several different bile acids, including CDCA and its glycine and taurine conjugates, bind and activate FXR at physiologic concentrations. Moreover, FXR response elements (FXREs) were identified in both the mouse and human *I-BABP* promoters (Grober et al., 1999; Makishima et al., 1999), which provided strong evidence that FXR mediates the positive effects of bile acids on *I-BABP* expression. Notably, the rank order of bile acids that activate FXR correlates with that for repression of *CYP7A1* in a hepatocyte-derived cell line (Makishima et al., 1999). These data suggested that FXR also has a role in the negative effects of bile acids on gene expression. However, since the region of the *CYP7A1* promoter that is necessary for bile acid–mediated repression lacks a strong FXR-binding site (Chiang and Stroup, 1994; Chiang et al., 2000), it seemed unlikely that this repression was a direct effect of FXR. Thus, the molecular mechanism for bile acid–mediated repression of *CYP7A1* remained in question.

In this report, we have used a potent, nonsteroidal FXR ligand to demonstrate that FXR regulates the hepatic expression of small heterodimer partner 1 (SHP-1; NR0B2), an atypical, orphan member of the nuclear receptor family that lacks a DNA-binding domain (Seol et al., 1996). SHP-1 has been shown to bind to other nuclear receptors and to repress their transcriptional activities (Seol et al., 1996; Masuda et al., 1997; Johansson et al., 1999; Lee et al., 2000). We show that SHP-1 represses the *CYP7A1* promoter through interaction with liver receptor homolog 1 (LRH-1; NR5A2), an orphan nuclear receptor that binds as a monomer to a response

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element in the *CYP7A1* promoter and activates transcription (Becker-Andre et al., 1993; Galameau et al., 1996; Nitta et al., 1999). LRH-1 is a mammalian homolog of the *Drosophila* fushi tarazu F1 gene product, which regulates *Drosophila* metamorphosis (Lavorgna et al., 1991; Broadus et al., 1999). Our findings define a novel regulatory cascade of three orphan nuclear receptors that provides a molecular basis for the coordinate repression of gene expression by bile acids.

Results

Identification of GW4064 as a Potent, Selective FXR Activator

FXR was recently shown to be a receptor for CDCA as well as other bile acids (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999). However, these compounds bind to FXR with only micromolar affinities and at these concentrations also interact with other proteins, including bile acid–binding proteins and transporters. We sought to identify a potent, selective FXR ligand for use as a chemical tool in elucidating the genes regulated by FXR. Combinatorial libraries of compounds were screened using a ligand-sensing fluorescence resonance energy transfer assay that detects interactions between FXR and a peptide derived from the steroid receptor coactivator 1 (SRC-1) as previously described (Parks et al., 1999). Among the compounds that promoted an interaction between FXR and SRC-1 was the isoxazole GW4064 (Figure 1A), which bound to FXR with a half-maximal effective concentration (EC_{50}) of 15 nM (Maloney et al., 2000). GW4064 activated mouse and human FXR with EC_{50} values of 80 and 90 nM, respectively, in CV-1 cells transfected with FXR expression vectors and a reporter plasmid containing two copies of an established FXR response element (FXRE) derived from the *Drosophila* heat shock protein 27 (hsp27) promoter (Forman et al., 1995) (Figure 1B). Thus, GW4064 is ~1000-fold more potent than CDCA in activating FXR in CV-1 cells (Figure 1B).

GW4064 was tested for selectivity against a panel of nuclear receptors. CV-1 cells were transfected with expression plasmids for various nuclear receptor–GAL4 chimeras and the reporter plasmid $(UAS)_5$-tk-CAT as previously described (Parks et al., 1999). GW4064 activated only the FXR-GAL4 chimera (Figure 1C). Thus, GW4064 is a highly selective activator of FXR.

FXR Regulates *SHP-1* Expression in the Liver

GW4064 was exploited as a chemical tool to identify the genes regulated by FXR in the liver. Male Fisher rats were treated for 7 days with GW4064 or vehicle alone (methyl cellulose). Following treatment, RNA was prepared from the livers of GW4064- and vehicle-treated animals, and genes that were either induced or repressed by GW4064 treatment were determined using CuraGen GeneCalling™ differential gene expression technology (Shimkets et al., 1999). A comprehensive list of the liver genes regulated by GW4064 will be published elsewhere. Interestingly, the gene that was most strongly induced by GW4064 treatment was that encoding the orphan nuclear receptor SHP-1. Northern analysis showed that *SHP-1* expression was increased ~6-fold in the livers of GW4064-treated rats relative to vehicle-treated animals (Figure 2A).

Bile acids are known to repress the expression of

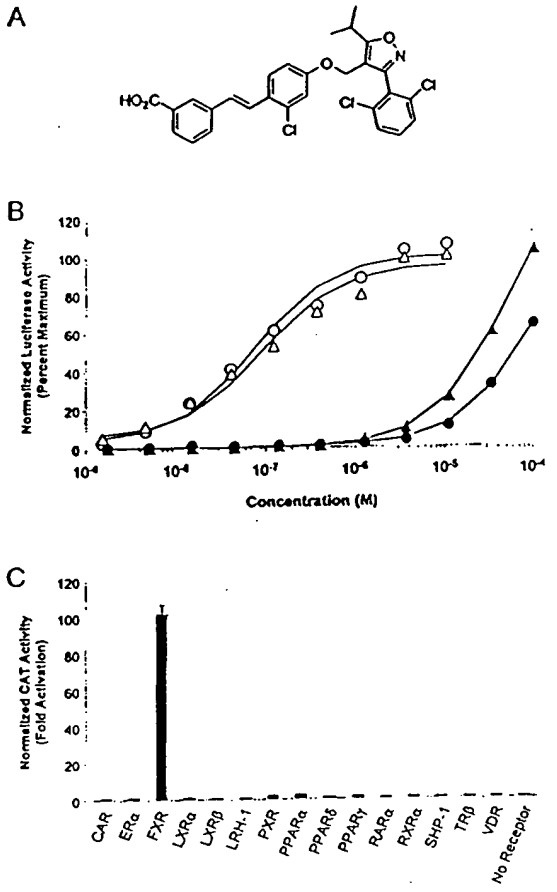

Figure 1. GW4064 Is a Potent, Selective Activator of FXR

(A) Chemical structure of GW4064.

(B) CV-1 cells were transfected with expression plasmids for human or mouse FXR and the $(hsp70EcRE)_3$-tk-LUC reporter plasmid containing two copies of the hsp70 ecdysone response element upstream of the thymidine kinase (tk) promoter and luciferase gene. Transfected cells were treated with the indicated concentrations of either GW4064 or CDCA. Open circles, mouse FXR and GW4064; open triangles, human FXR and GW4064; closed circles, mouse FXR and CDCA; closed triangles, human FXR and CDCA. Data points represent the mean of assays performed in triplicate.

(C) CV-1 cells were transfected with expression vectors for various GAL4–nuclear receptor ligand-binding domain chimeras and the reporter plasmid $(UAS)_5$-tk-CAT. Transfected cells were treated with 1 μM GW4064. Data represent the mean of assays performed in triplicate ± S.D.

CYP7A1 as part of a regulatory feedback loop that controls the rate of their biosynthesis from cholesterol (Russell and Setchell, 1992; Russell, 1999). Two recent studies implicate FXR in the repression of *CYP7A1* (Makishima et al., 1999; Wang et al., 1999), although the molecular mechanisms have remained unclear since the *CYP7A1* promoter does not contain a consensus FXRE (Chiang et al., 2000). In parallel with our analysis of *SHP-1* expression, we examined whether GW4064 treatment resulted in decreased *CYP7A1* expression in male Fisher rats. Rats treated with GW4064 showed a substantial decrease in *CYP7A1* mRNA levels (~4-fold, Figure 2A). Thus, GW4064 mimics the well documented

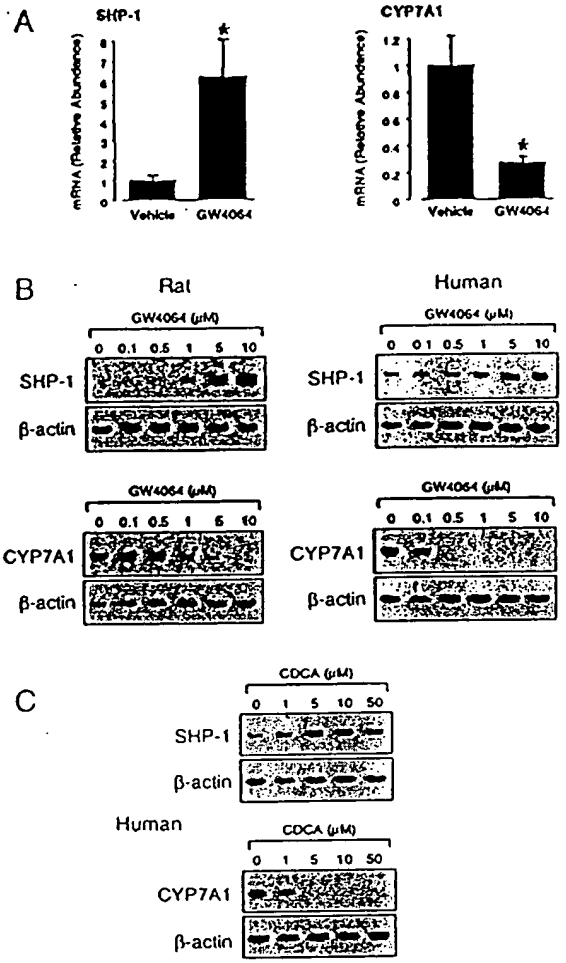

Figure 2. FXR Ligands Induce *SHP-1* and Repress *CYP7A1* Expression

(A) Total RNA was prepared from the livers of male Fisher rats treated for 7 days with GW4064 or vehicle alone. Northern analysis was performed using probes for rat *SHP-1* and *CYP7A1*. Data represent the mean ($n = 3$) ± standard error of the means. The asterisk denotes a statistically significant difference between vehicle- and GW4064-treated animals; $P < 0.05$.

(B) Total RNA was prepared from primary rat or human hepatocytes treated for 48 hr with the indicated concentrations of GW4064 or vehicle alone. Northern analysis was performed using probes for rat or human *SHP-1*, *CYP7A1*, or β-actin.

(C) Total RNA was prepared from primary human hepatocytes treated for 48 hr with the indicated concentrations of CDCA. Northern analysis was performed using probes for human *SHP-1*, *CYP7A1*, or β-actin.

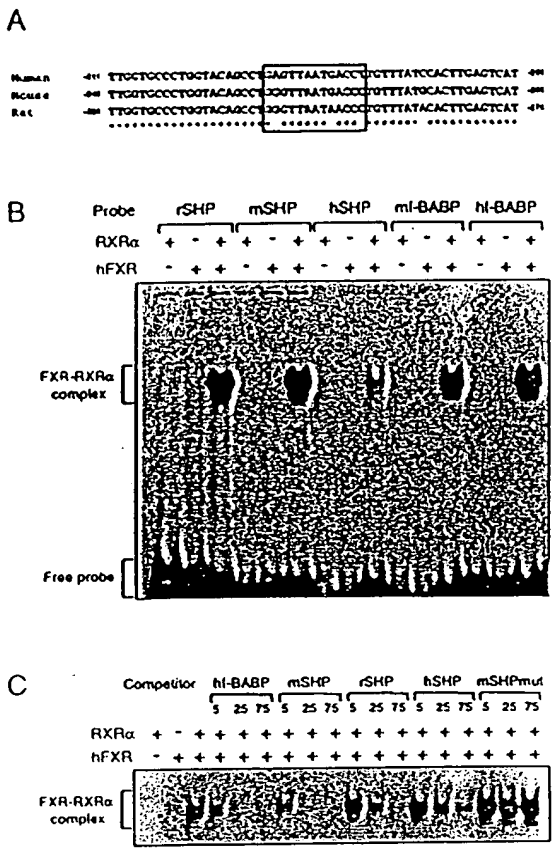

Figure 3. Identification of FXR Binding Sites in the Human, Rat, and Mouse *SHP-1* Promoters

(A) Alignment of the proximal regions of the human, rat, and mouse *SHP-1* promoters. The conserved IR1 FXR binding site is boxed. Conserved nucleotides are indicated by asterisks.

(B) Electrophoretic mobility-shift assays were performed with in vitro synthesized human FXR and/or human RXRα as indicated and [^{32}P]-labeled oligonucleotides containing the IR1 motif from the rat, mouse, or human *SHP-1* promoters or the mouse or human *I-BABP* promoters. The positions of the shifted FXR/RXRα complex and free probes are indicated.

(C) Electrophoretic mobility-shift assays were performed with in vitro synthesized human FXR and/or human RXRα, a [^{32}P]-labeled oligonucleotide containing the human *I-BABP* FXRE, and either a 5-, 25-, or 75-fold excess of unlabeled oligonucleotides containing the IR1 motifs from the human *I-BABP* promoter, the mouse, rat, or human *SHP-1* promoters, or a mutated derivative of the mouse *SHP-1* IR1 motif (mSHPmut). The position of the shifted FXR/RXRα complex is indicated.

effects of naturally occurring FXR ligands, namely bile acids, on *CYP7A1* expression. This observation provides compelling evidence that FXR mediates feedback repression of *CYP7A1* by bile acids.

To substantiate the in vivo data and extend them to human hepatocytes, we examined whether *SHP-1* and *CYP7A1* expression were regulated by FXR in primary cultures of rat and human hepatocytes. Hepatocytes were treated with increasing concentrations of GW4064, and the levels of *SHP-1* and *CYP7A1* expression were examined by Northern blot analysis. GW4064 treatment markedly increased *SHP-1* expression and decreased *CYP7A1* expression in hepatocytes from both species in a dose-dependent fashion (Figure 2B). Similar results were obtained in human hepatocytes treated with the natural FXR ligand CDCA (Figure 2C). As expected, CDCA was less potent than GW4064 in its effects on gene expression (compare Figures 2B and 2C). These data strongly suggest that FXR regulates *SHP-1* and *CYP7A1* expression in both human and rodent hepatocytes. Notably, there was a striking reciprocal relationship between the regulation of *SHP-1* and *CYP7A1*

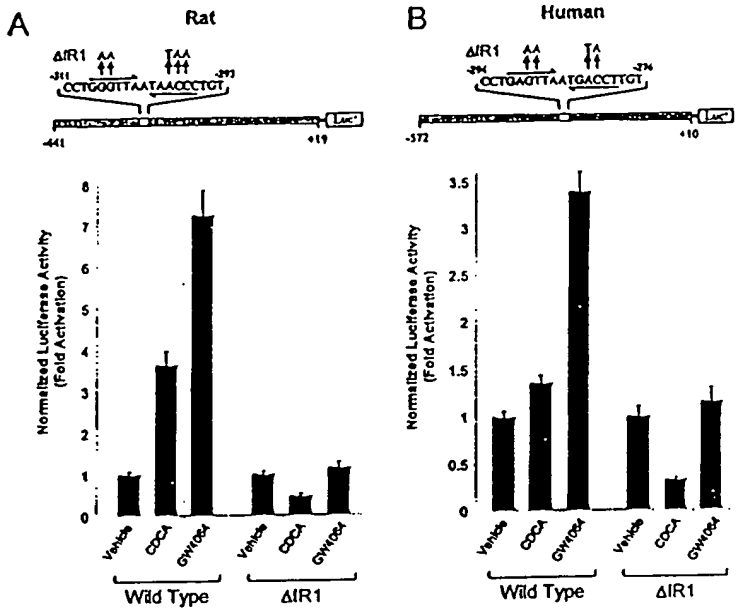

Figure 4. FXR Activates the Rat and Human *SHP-1* Promoters

HepG2 cells were transfected with the human FXR expression plasmid and luciferase reporter plasmids containing the proximal promoters of the rat ([A], nucleotides −441 to +19) or human ([B], nucleotides −572 to +10) *SHP-1* genes or the corresponding reporter plasmids in which the IR1 elements had been mutated (ΔIR1). Following transfection, cells were treated for 48 hr with GW4064 (1 μM) or CDCA (100 μM). Data represent the mean ± S.D. of six individual transfections.

expression: GW4064 and CDCA repressed *CYP7A1* expression at the same concentrations that were required to induce *SHP-1* expression (Figures 2B and 2C). Since SHP-1 is known to heterodimerize with several other members of the nuclear receptor superfamily and to repress their transcriptional activity (Seol et al., 1996; Masuda et al., 1997; Johansson et al., 1999), these data raised the intriguing possibility that FXR-mediated induction of SHP-1 might underlie the repression of *CYP7A1* expression (see below).

FXR Binds and Activates *SHP-1* Promoters

We next sought to determine whether *SHP-1* expression is directly regulated by FXR. FXR preferentially binds as a heterodimer with RXR to FXREs composed of two nuclear receptor half-sites of consensus AG(G/T)TCA organized as an inverted repeat and separated by a single nucleotide (IR1) (Forman et al., 1995). IR1-type FXREs have been identified in the human and mouse *I-BABP* promoters (Grober et al., 1999; Makishima et al., 1999). The mouse, rat, and human *SHP-1* promoters were examined for IR1 motifs. A highly conserved IR1-like element was identified ~300 nucleotides upstream of the transcription initiation site in the *SHP-1* promoter of all three species (Figure 3A). Electrophoretic mobility-shift analyses demonstrated that the FXR/RXR complex binds efficiently to the IR1 element from the *SHP-1* promoter of each species (Figure 3B). In agreement with earlier observations (Grober et al., 1999), the FXR/RXR heterodimer also bound to the mouse and human *I-BABP* FXREs (Figure 3B). Competition binding analyses showed that these interactions were specific: no competition was seen with a mutated derivative of the IR1 motif derived from the mouse *SHP-1* promoter (Figure 3C).

The presence of an FXR/RXR binding site suggested that the *SHP-1* gene is directly regulated by FXR. To test this hypothesis, HepG2 cells were transfected with an FXR expression plasmid and reporter plasmids expressing luciferase under the control of either the rat or human *SHP-1* promoters. GW4064 treatment of cells transfected with the FXR expression plasmid and either promoter construct resulted in a marked induction of reporter activity (Figures 4A and 4B). Based on Northern blot analysis of *SHP-1* expression (Figure 2B), the magnitude of the response from the rat (7-fold) and human (3-fold) *SHP-1* promoters was somewhat lower than expected and it is possible that other promoter or enhancer elements contribute to the regulation of *SHP-1* expression. Alternately, additional factors present in well differentiated cultures of rat hepatocytes but not HepG2 cells may be required for maximal FXR responsiveness. In the absence of exogenously expressed FXR, the rat and human *SHP-1* promoters exhibited a modest (~1.5-fold) induction on exposure to GW4064, which is most likely due to endogenous FXR in HepG2 cells (data not shown). FXR responsiveness was eliminated when mutations were introduced into the IR1 motifs in either the rat or human *SHP-1* promoters (Figures 4A and 4B). These data provide strong evidence that *SHP-1* expression is regulated directly by the FXR/RXR heterodimer in multiple species.

SHP-1 Interacts with Orphan Nuclear Receptor LRH-1

The finding that *SHP-1* expression is regulated by FXR together with the reciprocal relationship between *SHP-1* and *CYP7A1* regulation (Figure 2) suggested that SHP-1 might play a pivotal role in bile acid–mediated repression of *CYP7A1* expression. Regulation of the *CYP7A1* promoter is complex and involves numerous transcription factors, including nuclear receptors with known ligands such as the thyroid hormone receptor (TR), retinoic acid receptor (RAR), RXR and LXRα, and the orphan receptors COUP-TFII, HNF4α, and LRH-1 (Lehmann et al., 1997; Stroup et al., 1997; Chiang, 1998; Peet et al., 1998; Nitta et al., 1999; Russell, 1999; Stroup and Chiang, 2000). SHP-1 has previously been show to bind to and repress the transcriptional activities of TR, RAR, and RXR in the presence of their ligands and HNF4α in the

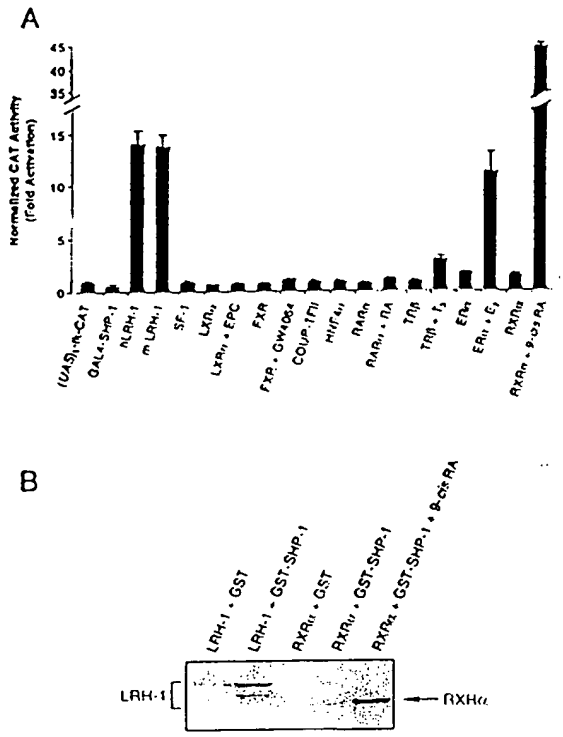

Figure 5. SHP-1 Interacts with the Orphan Nuclear Receptor LRH-1

(A) Mammalian two-hybrid experiments were performed in CV-1 cells cotransfected with expression plasmids for the GAL4-human SHP-1 chimera and various VP16–nuclear receptor ligand-binding domain chimeras. Transfection assays containing the LXRα-, FXR-, RARα-, TRβ-, ERα-, and RXRα-GAL4 chimeras were performed in the absence or presence of the indicated ligands (respectively: EPC, 24(S),25-epoxycholesterol (10 μM), GW4064 (1 μM); RA, all-*trans* retinoic acid (0.1 μM); T_3, triiodothyronine (0.1 μM); E_2, estradiol (0.1 μM); 9-*cis* RA, 9-*cis* retinoic acid (0.1 μM)]. Data are expressed as fold activation over cells transfected with the $(UAS)_5$-tk-CAT reporter alone and represent the mean of assays (n = 8) ± S.D.

(B) GST pull-down assays were performed with [^{35}S]-labeled LRH-1 or RXRα in the presence of GST or GST-SHP-1 as indicated. 9-*cis* retinoic acid (9-*cis* RA) was added to the binding reaction to a final concentration of 10 μM.

absence of any exogenous ligand (Seol et al., 1996; Masuda et al., 1997). Using a mammalian two-hybrid approach, we examined whether SHP-1 interacts with these and other nuclear receptors that have been implicated in the regulation of *CYP7A1*. CV-1 cells were transfected with an expression plasmid for a GAL4-SHP-1 chimera, the $(UAS)_5$-tk-CAT reporter, and expression plasmids for chimeras between the strong transcriptional activation domain of VP16 and the isolated ligand-binding domains of a panel of nuclear receptors (Figure 5A). When transfected alone, the GAL4-SHP-1 chimera caused a minor reduction (~0.3-fold) in reporter activity (Figure 5A). However, reporter activity was strongly induced when GAL4-SHP-1 was coexpressed with VP16-RXRα (~44-fold) or VP16-estrogen receptor α (ERα, ~11-fold) in the presence of 9-*cis* retinoic acid and estradiol, respectively (Figure 5A). These interactions were strongly dependent on the presence of ligand. Little or no interaction was detected between SHP-1 and LXRα, FXR, COUP-TFII, HNF4α, RARα, or TRβ in our mammalian two-hybrid assay (Figure 5A). The lack of a stronger interaction between SHP-1 and either TRβ, RARα, or HNF4α was surprising in light of the previous results of others (Seol et al., 1996; Masuda et al., 1997) and may reflect differences in the assay systems used. Notably, strong reporter activity was detected when GAL4-SHP-1 was expressed with VP16-human LRH-1 or VP16-mouse LRH-1 (~14-fold activation for both human and mouse). This activity was completely dependent on the presence of GAL4-SHP-1 (data not shown). These data demonstrate that SHP-1 can interact with LRH-1 in cells. Interestingly, little or no interaction was detected between SHP-1 and steroidogenic factor 1 (SF-1) (Figure 5A), a closely related orphan receptor that shares ~60% amino acid identity with LRH-1 in the ligand-binding domain (Tsukiyama et al., 1992; Honda et al., 1993; Ikeda et al., 1993).

Using a glutathione S-transferase (GST) pull-down assay, we examined whether SHP-1 binds directly to LRH-1. SHP-1 was expressed in *E. coli* as a fusion protein with GST, and [^{35}S]-labeled LRH-1 was synthesized in vitro. Glutathione–Sepharose beads efficiently coprecipitated [^{35}S]-labeled LRH-1 in the presence of GST-SHP-1 but not in its absence (Figure 5B). In parallel incubations, GST-SHP-1 interacted strongly with [^{35}S]-labeled human RXRα in the presence of 9-*cis* retinoic acid (Figure 5B). These data are in close agreement with those derived from mammalian two-hybrid experiments (Figure 5A). Thus, SHP-1 interacts directly with LRH-1.

SHP-1 Represses Expression of *CYP7A1*

Does SHP-1 have a role in the repression of *CYP7A1* expression by FXR ligands? We addressed this question by performing cotransfection experiments with a rat *CYP7A1* luciferase reporter plasmid (pGL3-rCYP7A1 [−1573/+36]) containing nucleotides −1573 to +36 of the rat *CYP7A1* promoter, which includes a conserved LRH-1 binding site (Nitta et al., 1999). In the absence of exogenously expressed LRH-1, the activity of the pGL3-rCYP7A1(−1573/+36) reporter was low when transiently transfected into HepG2 cells (data not shown). Cotransfection of increasing amounts of an LRH-1 expression plasmid resulted in a dose-dependent increase in reporter activity (Figure 6). This LRH-1-dependent reporter activity was completely blocked by the cotransfection of SHP-1 expression plasmid (Figure 6). These data suggest that interactions between SHP-1 and LRH-1 represent a basis for bile acid–mediated repression of *CYP7A1* expression.

Discussion

The recent discovery that FXR is a bile acid receptor provided a great deal of insight into the molecular mechanisms underlying bile acid signaling. In particular, these studies uncovered the mechanism whereby bile acids stimulate the transcription of genes, such as *I-BABP*, involved in bile acid transport. High-affinity binding sites for the FXR/RXR heterodimer have been identified in both the human and mouse *I-BABP* promoters (Grober et al., 1999; Makishima et al., 1999). By contrast, the mechanism underlying bile acid–mediated repression of *CYP7A1* expression remained a puzzle, since an FXRE had not been identified in the bile acid response elements of this gene (Chiang and Stroup, 1994; Chiang et

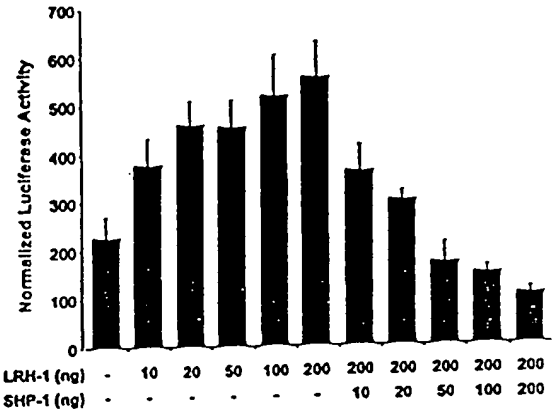

Figure 6. SHP-1 Represses LRH-1-Dependent Activation of the Rat *CYP7A1* Promoter

HepG2 cells were transfected with the rat *CYP7A1* reporter plasmid, pGL3-rCYP7A1(−1573/+36), and the indicated amounts of LRH-1 and/or SHP-1 expression plasmids. Data represent the mean of assays performed in triplicate ± S.D.

al., 2000). We now present evidence that FXR does not repress *CYP7A1* expression directly, but rather through induction of the gene encoding the orphan nuclear receptor SHP-1, which, in turn, represses *CYP7A1* expression. Similar findings have been reported by Lu et al. (2000 [this issue of *Mol. Cell*]). Consistent with this model, it was recently shown that *SHP-1* expression is markedly lower and not inducible by cholic acid in the livers of mice lacking FXR (Sinal et al., 2000). Taken together, these data provide a molecular explanation for the coordinate suppression of gene expression by bile acids.

SHP-1 Represses *CYP7A1* Expression

We encountered the orphan nuclear receptor SHP-1 as part of a comprehensive, unbiased effort to identify FXR target genes in the liver. *SHP-1* expression was strongly induced in the livers of rats treated with the potent, nonsteroidal FXR ligand GW4064. *SHP-1* expression was also markedly induced by GW4064 in primary cultures of human and rat hepatocytes, whereas *CYP7A1* expression was suppressed under the same conditions. The reciprocal relationship between *SHP-1* and *CYP7A1* regulation, together with the established inhibitory effects of SHP-1 on nuclear receptor activity, suggested that SHP-1 might repress *CYP7A1* expression. Indeed, expression of SHP-1 repressed the activity of the rat *CYP7A1* promoter in HepG2 cells.

SHP-1 is unusual in that it lacks the highly conserved DNA-binding domain typically found in members of the nuclear receptor family. SHP-1 was originally cloned in yeast two-hybrid experiments using the orphan nuclear receptors CAR or PPARα as bait, but it interacts with a number of additional nuclear receptors, including ERα and ERβ, RAR, RXR, and TR (Seol et al., 1996; Masuda et al., 1997; Seol et al., 1998; Johansson et al., 1999). In each case, SHP-1 represses the ligand-induced transcriptional activity of these receptors. How does SHP-1 repress transcription of the *CYP7A1* promoter? Our data indicate that SHP-1 exerts much of its effect through interaction with the orphan nuclear receptor LRH-1. SHP-1 interacted strongly with LRH-1 in both a mammalian two-hybrid assay and an in vitro pull-down assay. Moreover, SHP-1 efficiently repressed LRH-1-dependent activation of the rat *CYP7A1* promoter. LRH-1 was recently shown to activate the human *CYP7A1* promoter by binding to an extended nuclear receptor half-site sequence that is conserved in the mouse, rat, and hamster *CYP7A1* promoters (Nitta et al., 1999). Earlier studies had defined DNA response elements in the *CYP7A1* and *CYP8B1* gene promoters that conferred repression in response to bile acids (Chiang and Stroup, 1994; Chiang et al., 2000; del Castillo-Olivares and Gil, 2000). Notably, each of these negative bile acid response elements contains an LRH-1 binding site. Consistent with these data, *CYP8B1* expression was repressed 3-fold in Fisher rats treated with GW4064 (S. A. J., unpublished data). Thus, interactions between SHP-1 and LRH-1 are likely to be important for the coordinate repression of a number of genes by bile acids. Among the genes that may be regulated by the interaction between SHP-1 and LRH-1 is *SHP-1* itself. An LRH-1-responsive region of the murine *SHP-1* gene has been identified (Lee et al., 1999). Thus, SHP-1 is likely to regulate its own expression. This feedback regulation may provide a mechanism for attenuating the bile acid–mediated repression of genes by SHP-1. A model for bile acid–mediated repression of gene expression via increased SHP-1 levels is shown in Figure 7.

Two recent reports showed that SHP-1 represses the transcriptional activation of ERα and ERβ, RXR, and the orphan receptor HNF4α by competing with coactivator binding to these receptors (Johansson et al., 1999; Lee et al., 2000). In addition, SHP-1 contains a strong transcriptional repressor domain in its C terminus (Lee et al., 2000). Furthermore, SHP-1 has been shown to inhibit DNA binding of RAR-RXR heterodimers (Seol et al., 1996). Taken together, these studies suggest that SHP-1 inhibits the transcriptional activity of nuclear receptors through multiple mechanisms. To date, we have been unable to demonstrate inhibition of LRH-1 binding to its response element in the *CYP7A1* promoter by SHP-1 (data not shown). Thus, the mechanism by which SHP-1

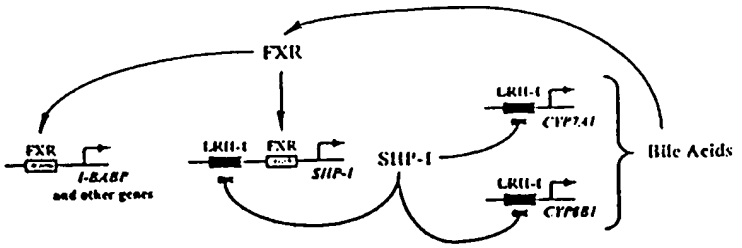

Figure 7. Model for the Feedforward and Feedback Regulatory Effects of Bile Acids on Gene Expression

Activation of FXR by bile acids results in the induction of *I-BABP* and *SHP-1* expression. SHP-1, in turn, interacts with LRH-1 and represses expression of *CYP7A1* and *CYP8B1*. SHP-1 may also repress expression of its own gene.

Inhibits LRH-1-mediated transactivation of the *CYP7A1* promoter remains unresolved.

In addition to the interactions between SHP-1 and LRH-1, other mechanisms may play a role in bile acid-mediated repression of *CYP7A1* expression. First, SHP-1 binds to and represses the transcriptional activity of other nuclear receptors that regulate *CYP7A1*, including RXR and TR (Seol et al., 1996; Masuda et al., 1997). These interactions may also contribute to bile acid-mediated repression of *CYP7A1* expression. Second, ligand-bound FXR was reported to repress LXRα activity on an LXRα response element (Wang et al., 1999), although the mechanism for this *trans*-repression is not clear. Since LXRα stimulates rodent *CYP7A1* expression in response to oxysterols, repression of LXRα activity may contribute to the overall repression of *CYP7A1*. Thus, SHP-1/LRH-1 interactions may be one of several mechanisms whereby bile acids repress expression of *CYP7A1* and other genes.

Parallels between SHP-1/LRH-1 and Other Nuclear Receptor Pairs

Intriguing parallels exist between the SHP-1/LRH-1 interaction and another pair of nuclear receptors. LRH-1 is most closely related to the orphan receptor SF-1, which regulates the expression of enzymes required for steroid hormone biosynthesis (Parker, 1998; Hammer and Ingraham, 1999). SF-1 and LRH-1 are ~85% identical in the amino acid sequences of their DNA-binding domains, and both bind as monomers to the same extended nuclear receptor half-site sequence. Notably, the transcriptional activity of SF-1 is repressed by binding to DAX-1 (dosage-sensitive sex-reversal adrenal hypoplasia congenital region on the X chromosome, region 1; NR0B1), an orphan nuclear receptor most closely related to SHP-1 that also lacks the DNA-binding domain characteristic of nuclear receptors (Zanaria et al., 1994; Hammer and Ingraham, 1999). Thus, both SF-1 and LRH-1 are negatively regulated in a *trans*-dominant fashion by heterodimerization with orphan receptors lacking DNA-binding domains. Since SHP-1 expression is stimulated by bile acids, it will be interesting to determine whether DAX-1 expression is also regulated by hormones.

A second nuclear receptor pair with similarities to SHP-1/LRH-1 occurs in *Drosophila*. Hormonal activation of the ecdysone receptor (EcR) during the third larval instar phase of *Drosophila* metamorphosis results in an increase in the expression of two orphan nuclear receptors, DHR3, which has a functional DNA-binding domain, and E75B, which does not. E75B binds to DHR3 and represses its transcriptional activity (Thummel, 1997; White et al., 1997). This interaction is critical for determining the temporal progression of metamorphosis. The EcR/E75/DHR3 and FXR/SHP-1/LRH-1 regulatory cascades are remarkably similar in that hormone-mediated activation of a nuclear receptor (either FXR or EcR) induces expression of a second nuclear receptor, which, in turn, binds to and represses the activity of a third nuclear receptor. The similarities in these genetic hierarchies across evolution suggest that repression via heterodimerization may represent an important paradigm for the modulation of orphan receptor activity.

Conclusions

The mechanism whereby FXR represses expression of *CYP7A1* and other genes has until now remained an enigma. Through the use of a potent, nonsteroidal FXR ligand, we have identified *SHP-1* as an FXR target gene in the liver of humans and rodents. Furthermore, we have demonstrated that SHP-1 can interact with LRH-1 and efficiently repress expression of *CYP7A1*. Thus, bile acid–induced repression of *CYP7A1* is mediated by a novel regulatory cascade of three nuclear receptors. Since both the *CYP7A1* and *CYP8B1* gene promoters contain LRH-1 binding sites, the SHP-1/LRH-1 partnership is likely to have broad implications in bile acid signaling. Both SHP-1 and LRH-1 are orphan receptors, which raises the possibility that bile acid biosynthesis will be regulated by additional, unidentified hormones. Regardless of whether SHP-1 and LRH-1 have natural ligands, pharmacologic modulation of their interaction represents an exciting new opportunity for the discovery of drugs that regulate cholesterol homeostasis.

Experimental Procedures

Materials

The synthesis of GW4064 will be described elsewhere (Maloney et al., 2000). CDCA, dexamethasone, estradiol, all-*trans* retinoic acid, 9-*cis* retinoic acid, and charcoal-stripped, delipidated calf serum were acquired from the Sigma Chemical Co. (St. Louis, MO). 24(S),25-epoxycholesterol was synthesized in-house. DNA-modifying enzymes, polymerases, and restriction endonucleases were provided by Roche Molecular Biochemicals (Indianapolis, IN). Charcoal/dextran-treated fetal bovine serum (FBS) was purchased from Hyclone Laboratories Inc. (Logan, UT). The human hepatocellular carcinoma cell line HepG2 was obtained from the American Type Culture Collection (ATCC number HB-8065, Manassas, VA). Matrigel was provided by Becton Dickinson Labware (Bedford, MA). All other tissue culture reagents were obtained from Life Technologies Inc. (Gaithersburg, MD).

Animals

Male Fisher rats were obtained from Charles River Laboratories Inc. (Raleigh, NC) and maintained on a 12 hr light/12 hr dark cycle. Animals were allowed food and chow ad libitum. GW4064 (30 mg/kg) was administered by gavage twice a day for 7 days and the animals sacrificed by cervical dislocation 4 hr after the final treatment. Livers were excised and snap-frozen in liquid nitrogen. Differential gene expression analysis was performed by CuraGen Corp. (New Haven, CT).

Plasmid Constructs

Expression plasmids for the human nuclear receptor–GAL4 chimeras were prepared by inserting amplified cDNAs encoding the ligand-binding domains into a modified pSG5 expression vector (Stratagene, La Jolla, CA) containing the GAL4 DNA-binding domain (amino acids 1–147) and the Simian virus 40 (SV40) large T antigen nuclear localization signal (APKKKRKVG). The $(UAS)_4$-tk-CAT and $(hsp27EcRE)_2$-tk-LUC reporter constructs have been previously described (Forman et al., 1995; Parks et al., 1999). pβ-actin-SPAP, an expression vector containing the human secreted placental alkaline phosphatase (SPAP) cDNA under the control of β-actin promoter, was used as an internal control in all transfections. The expression plasmids for human and mouse FXR (pSG5-hFXR and pSG5-mFXR, respectively) and human SRC-1 are described elsewhere (Kliewer et al., 1998; Parks et al., 1999). The full-length coding regions for human LRH-1 (GenBank Accession Number AB019246) and human SHP-1 (GenBank Accession Number L76571) were amplified by PCR and cloned into pSG5, creating pSG5-hLRH-1 and pSG5-hSHP-1, respectively. A consensus Kozak sequence was created during amplification. The rat (bases −441 to +19, GenBank Accession Number D86745) (Masuda et al., 1997) and human (bases −572 to +10, GenBank Accession Number AF044316) (Lee et al., 1998) *SHP-1* promoters were amplified by PCR using the following primer pairs: Rat, 5′-gggtgtgcgagatctCCCTGGCTGGCTCCTTGGCTCTGT-3′ (sense) and 5′-gggtgtgcgagatctCCTGTTTCTTCCTGGCTCTGT

GGC-3′ (antisense); and human, 5′-gggtgtgcgagatctTCCTAGACT GGACAGTGGGCAAAG-3′ (sense) and 5′-gggtgtgcgagatctCTTCC AGCTCTCTGGCTCTGTGTT-3′ (antisense). The resultant fragments were inserted into the *Bgl*II site of pGL3-Basic, a promoter-less luciferase reporter vector (Promega, Madison, WI). Site-directed mutagenesis of putative FXREs in the rat and human *SHP-1* promoters was performed using the Transformer mutagenesis system (CLONTECH Laboratories, Palo Alto, CA) with the ΔratIR1 (bases −321 to −287, 5′-CCTGGTACAGCCTGGaaTAATAtaaCTGTTTATAC-3′) and ΔhumanIR1 (bases −304 to −270, 5′-CCTGGTACAGCCTGA aaTAATGtaCTTGTTTATCC-3′) primers. Mutated constructs were verified to be free of nonspecific base changes by sequencing. pGL3-rCYP7A1(−1573/+36) contains bases −1573 to +36 of the rat *CYP7A1* promoter (GenBank Accession Number Z14108) inserted into the *Nhe*I site of pGL3-Basic. VP16-nuclear receptor chimeras contain the 80 aa Herpes virus VP16 transactivation domain linked to the ligand-binding domain of the following nuclear receptors in a modified pSG5 expression vector: human COUP-TFII, ERα, LRH-1, LXRα, RARα, and TRβ; mouse FXR, LRH-1, RXRα, and SF-1; and rat HNF4α.

Transient Transfection Assays

Transient transfection of CV-1 cells was performed exactly as described elsewhere (Jones et al., 2000). Typically, transfection mixes contained 2–5 ng of receptor expression vector, 20 ng of reporter construct, and 8 ng of pβ-actin-SPAP. The amount of DNA used in each transfection was adjusted to 80 ng with carrier plasmid (pBluescript, Stratagene). Mammalian two-hybrid experiments utilized transfection mixes containing 20 ng of VP16 nuclear receptor ligand-binding domain expression vector, 5 ng of pSG5-GAL4-SHP-1, 15 ng of $(UAS)_5$-tk-CAT, and 8 ng of pβ-actin-SPAP. Cells were maintained for 24 hr in the presence of drug (added as a 1000× stock in dimethyl sulfoxide) in DMEM/F-12 nutrient mixture containing 10% charcoal-stripped, delipidated calf serum. An aliquot of medium was assayed for SPAP activity, and the cells were lysed prior to determination of luciferase expression. Luciferase activities were normalized to SPAP. HepG2 cells were maintained in DMEM/F-12 supplemented with 10% heat-inactivated FBS (Life Technologies Inc.). Plasmid DNA was transfected into HepG2 cells using the FuGENE6 transfection reagent according to the manufacturer's instructions (Roche Molecular Biochemicals). Thus, 24-well culture plates (15 mm diameter) were inoculated with 7×10^5 cells 24 hr prior to transfection. Cells were transfected overnight in serum-free DMEM/F-12 with 100 ng of reporter construct, 32 ng of pβ-actin-SPAP, and 0–400 ng of receptor expression vectors (adjusted to 400 ng with carrier plasmid). Following transfection, the medium was aspirated and the cells were cultured for a further 48 hr in DMEM/F-12 supplemented with 10% heat-inactivated FBS. SPAP and luciferase values were determined as described above.

Primary Culture of Human and Rat Hepatocytes and Northern Blot Analysis

Primary human hepatocytes were obtained from Dr. Steve Strom (University of Pittsburgh). Rat hepatocytes were isolated as described elsewhere (LeCluyse et al., 1996). Cells (1.5×10^6) were cultured on Matrigel-coated 6-well plates in serum-free Williams' E medium supplemented with 100 nM dexamethasone, 100 U/ml penicillin G, 100 μg/ml streptomycin, and insulin-transferrin-selenium (ITS-G, Life Technologies Inc.). Twenty-four hours after isolation, hepatocytes were treated with either GW4064 (0.1–10 μM) or CDCA (1–100 μM), which were added to the culture medium as 1000× stocks in dimethyl sulfoxide. Control cultures received vehicle alone. Cells were cultured for a further 48 hr prior to harvest, and total RNA was isolated using a commercially available reagent (Trizol, Life Technologies Inc.) according to the manufacturer's instructions. Total RNA (10 μg) was resolved on a 1% agarose/2.2 M formaldehyde denaturing gel and transferred to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech Inc., Piscataway, NJ). Blots were hybridized with ^{32}P-labeled cDNAs corresponding to human *SHP-1* (GenBank Accession Number L76571), human *CYP7A1* (bases 99–1564, GenBank Accession Number M93133), mouse *SHP-1* (bases 30–783, GenBank Accession Number L76567), or rat CYP7A1 (bases 235–460, GenBank Accession Number J05460). Subsequently, blots were stripped and reprobed with a radiolabeled β-actin cDNA (CLONTECH Laboratories).

Electrophoretic Mobility-Shift Assays

Electrophoretic mobility-shift assays (EMSA) were performed essentially as described elsewhere (Lehmann et al., 1997). hFXR and hRXRα were synthesized from pSG5-hFXR and pSG5-hRXRα expression vectors, respectively, using the TNT T7 Coupled Reticulocyte System (Promega). Unprogrammed lysate was prepared using the pSG5 expression vector (Stratagene). Binding reactions contained 10 mM HEPES (pH 7.8), 60 mM KCl, 0.2% Nonidet P-40, 6% glycerol, 2 mM dithiothreitol (DTT), 2 μg of poly(dI-dC)•poly(dI-dC), and 1 μl each of synthesized hFXR or hRXRα. Control incubations received unprogrammed lysate alone. Reactions were preincubated on ice for 10 min prior to the addition of [^{32}P]-labeled double-stranded oligonucleotide probe (0.2 pmol). Competitor oligonucleotides were added to the preincubation at 5-, 25-, and 75-fold molar excess. Samples were held on ice for a further 20 min, and the protein–DNA complexes resolved on a pre-electrophoresed 5% polyacrylamide gel in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA) at room temperature. Gels were dried and autoradiographed at −70°C for 1–2 hr. The following double-stranded oligonucleotides were used as probes and competitors in EMSA: rSHP, 5′-gatcCCTG GGTTAATAACCCTGT-3′; mSHP, 5′- gatcCCTGGGTTAATGACCC TGT-3′; hSHP, 5′- gatcCCTGAGTTAATGACCTTGT-3′; mI-BABP, 5′-gatcTTAAGGTGAATAACCTTGG-3′; hI-BABP, 5′-gatcCCAGGT GAATAACCTCGG-3′ (Grober et al., 1999); and mSHPmut 5′-gatcCC TGGaaTAATGttCCTGT-3′.

GST Pull-Down Assays

GST-SHP-1 fusion protein was expressed in BL21(DE3)plysS cells and bacterial extracts prepared by one cycle of freeze-thaw of the cells in protein lysis buffer containing 50 mM Tris (pH 8.0), 250 mM KCl, 1% Triton X-100, 10 mM DTT and 1× Complete Protease Inhibitor (Roche Molecular Biochemical) followed by centrifugation at 40,000 × g for 30 min. Glycerol was added to the resultant supernatant to a final concentration of 10%. Lysates were stored at −80°C until use. [^{35}S]-labeled human LRH-1 or human RXRα was generated using TNT T7 Coupled Reticulocyte System (Promega) in the presence of Pro-Mix (Amersham Pharmacia Biotech Inc.). Coprecipitation reactions included 25 μl of lysate containing GST-SHP-1 fusion protein or control GST; 25 μl of incubation buffer (50 mM KCl, 40 mM HEPES [pH 7.5], 5 mM β-mercaptoethanol, 0.1% Tween 20 and 1% nonfat dry milk); and 5 μl of [^{35}S]-labeled LRH-1 or RXRα. The mixtures were, incubated for 25 min with gentle rocking at 4°C prior to the addition of 20 μl of glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech Inc.) that had been extensively washed in protein lysis buffer. Reactions were incubated at 4°C with gentle rocking for a further 20 min. The beads were pelleted at 3000 rpm in a microfuge and washed four times with protein incubation buffer. Following the final wash, the beads were resuspended in 25 μl of 2× SDS-PAGE sample buffer containing 50 mM DTT. Samples were heated to 100°C for 5 min and resolved on a 10% acrylamide gel. Autoradiography was performed overnight.

Statistical Analyses

Unless otherwise stated, data are expressed as mean ± standard deviation (S.D.). The significance of differences in *SHP-1* and *CYP7A1* expression between vehicle- and GW4064-treated animals were analyzed using an unpaired Student's *t*-test.

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APPENDIX D

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PHARMACOLOGICAL REVIEWS

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The Classification of Seven Transmembrane Receptors in Recombinant Expression Systems

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'. . . . our complicated experiments have no longer anything to do with nature in her own right, but with nature changed and transformed by our own cognitive activity.' Werner Heisenberg (1901–1976)

I. Introduction

The major premise of this review is that seven transmembrane receptors (7 transmembrane (TM) receptors, also referred to in literature as G-protein coupled receptors) are specialized proteins designed for chemical *recognition* of ligands and the subsequent *transduction* of the information encoded in those ligands to the machinery of the cell (Kenakin et al., 1992). The superfamily of 7TM domain G-protein coupled receptors interact with alkaloids, biogenic amines, peptides, glycoprotein hormones, light and odorants. They are unsurpassed as therapeutic targets and probably will continue to be so

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in the future. For example, it can be estimated that if only 5% of the human genome codes for 7TM receptors, then there is complementary deoxyribonucleic acid (cDNA) available for 5000 receptors. Less than 10% of these are known leaving a fertile area for drug development. To this may be added also the potential targets of 7TM receptor mutations in disease states.

To achieve information transfer, the ability to bind ligands to a recognition domain and allosterically transmit the presence of that ligand to an intracellular domain appears to be a specialized feature of 7TM receptors. The very properties that define receptors as such also impart unique protein behaviors to receptors, and these behaviors, in turn, affect drug activity.

Heterologously expressed receptors and combinatorial libraries of molecules represent the new technology of drug discovery (Luyten et al., 1991; Luyten and Leysen, 1993; Baum, 1994). Until the advent of molecular biology, the behavior of receptors was limited by the constrained stoichiometries of natural systems. However, the ability to insert receptors into foreign surrogate cells at various expression levels has led to an explosion of information regarding the activity of drugs and the behavior of receptors. This has led to better understanding of the effects of receptor-effector stoichiometry and the influence of the cellular host on receptors. The behavior of receptor proteins can be critical to what is observed as drug-induced activity. This review discusses the recognition of aberrant receptor behavior in foreign cells and the measurement of drug-receptor parameters that transcend this behavior.

II. Receptor Pharmacology in Drug Discovery

For most of the history of receptor pharmacology, the discovery of biologically active ligands has centered on the testing of chemicals on animal host systems containing teleologically 'similar' receptors. This similarity generally has stemmed from the fact that the endogenous chemicals to be recognized often are the same in both animals and humans (i.e., neurotransmitters such as norepinephrine, acetylcholine, etc.). It followed, therefore, that the recognition units for these chemicals (the receptors) could be similar enough to detect like activity that would transcend the gap between animal and human host systems. The science of pharmacology has been built upon this correspondence.

Recent advances in molecular biology have greatly reduced the need for reliance on animal receptor systems and allowed the critical testing of this approach. Thus, the steps toward total correspondence (i.e., the testing of drugs on the human receptor in the exactly correct tissue under the appropriate pathology) have been made with advances in molecular biology (see table 1). Currently, the state of the art mainly resides in systems where human receptor material (i.e., cDNA) coding for receptor is introduced into surrogate cells.

While animal receptor systems are available and can be considered 'physiological,' the obvious shortcomings of such systems are the fact that the receptors are still facsimiles of the human targets. Another problem with natural animal systems is related to their basic design. There is evidence to suggest that, perhaps as a response to the need to finely tune the control of cellular biochemistry, cells express mixtures of receptor subtypes in varying quantities to take advantage of endogenous agonist information. This results in the study of ligands on mixtures of very similar binding sites, leading to the obvious potential for misleading classification. The expression of human receptors in surrogate cell systems has eliminated these shortcomings, i.e., human receptors can be expressed in apparently (vide infra) pure populations in cells.

There is a good deal of circumstantial evidence available to suggest that receptors from animal sources are good templates for predicting drug activity on human receptors. However, there also is striking evidence that slight differences between human and animal receptors can have profound effects on drug activity. It is known that there are differences in affinity that result from relatively small sequence differences between human and animal receptors, as seen in the rat and human 5-hydroxytryptamine $(5\text{-HT})_{2A}$ receptor (Johnson et al., 1994). This is especially true for nonpeptide antagonists for peptide receptors where it appears that evolution has produced mutations that have not altered binding of

Abbreviations: TM, transmembrane; cDNA, complementary deoxyribonucleic acid; 5-HT, 5-hydroxytryptamine; mRNA, messenger ribonucleic acid; T, population of 7TM receptors that can exist in a so-called 'inactive' state T (following the convention for ion channels); R, population of 7TM receptors that can exist in a so-called 'active' state; L, allosteric constant (where L = [T]/[R]); A, a drug; M, ability of A to alter equilibrium; cAMP, cyclic adenosine monophosphate; CGRP, calcitonin gene related peptide; ASPET, American Society for Pharmacology and Experimental Therapeutics; PI, phosphoinositol; XAC, [^{3}]xanthine amine congener; PKC, protein kinase C; GTP, guanosine triphosphate; DADLE, [D-Ala^2, D-Leu^5]enkephalin; GnRH, gonadotrophin-releasing hormone; GDP, guanosine diphosphate; CCK, cholecystokinin; NECA, 5'-N-ethylcarboxamidoadenosine; m1AChR, m1 acetylcholine receptors; PACAP, pituitary adenylate cyclase-activating polypeptide; PTX, pertussis toxin; CTX, cholera toxin; SPAP, secreted human placental alkaline phosphatase.

TABLE 1
Pharmacological receptor testing systems

Animal receptors-animal tissues
↓
Animal genetic receptor material-animal surrogate cells
↓
Human genetic receptor material-animal surrogate cells
↓
Human genetic receptor material-human surrogate cells
↓
Human genetic receptor material-human target cells
↓
Human genetic receptor material-human target cells with appropriate pathology

natural peptides but do produce differences for foreign nonpeptide ligands (Jensen et al., 1994). Thus, two recently developed nonpeptide substance-P antagonists show marked differences in their affinity for human substance-P receptors as compared with the corresponding rat receptor (Fong et al., 1992b). In some cases, differences in affinity for ligands may result from very small differences in amino acid sequence as in the *single* amino acid difference between the human and rat 5-HT_{1B} receptor (Oksenberg et al., 1992; Metcalf et al., 1992). The presence of $threonine_{355}$ in the human 5-HT_{1B} receptor (as opposed to a corresponding asparagine in the rat receptor) accounts for a remarkably different pharmacology between the two receptors, despite a 95% amino acid sequence identity (Hamblin et al., 1992).

In general, it is not possible to prove that differences in amino acid sequences in receptors will *not* result in different pharmacology because the differences may be ligand-specific. For example a single point mutation in human 5-HT receptors (5-$HT_{1D\alpha}$, 5-HT_{1F}) increases the affinity for propranolol and pindolol by a factor of 100- to 1000-fold but leaves the affinity for 5-HT unchanged (Adham et al., 1994a).

There are specific cases in which receptors from animal sources would not be predictive to human disease. For example, polymorphic variations in human dopamine D_4 receptors, thought to be related to psychiatric disorders, result in receptors with variably sized third cytoplasmic loops. Because this region of the human receptor is not found in the rat homologue of the D_4 receptor, the rat receptor would not reflect ligand-specific effects in this human population (Van Tol et al., 1992). For these reasons, it is obvious that the testing of possible new drug entities on human receptors is preferred.

III. Translation, Expression, and Co- or Post-translational Modification

It must be assumed that the genetic material introduced into the surrogate cell can find its way to the appropriate locus, be translated correctly and the resulting product processed as in native systems. The correct transcription of the gene in the expression system may be critical to subsequent expression. For example, single site-directed mutagenesis has shown that prevention of a putative cysteine-cysteine disulfide bond in the γ-aminobutyric acid type A channel prevents the functional expression of that receptor subunit (Amin et al., 1994). Complete sequences must be expressed for correct receptor function. Truncates of receptors, when compared with full-length wild type receptor, have been shown to have lower affinity (Fong et al., 1992a), no differences in affinity (Rodriguez et al., 1992; Reneke et al., 1988) or increased affinity (Findlay et al., 1994). In general, there is considerable evidence that nonstandard translational events may affect the nature of expression products (Santos and Tuite, 1993).

Expression of multiunit receptors can be especially difficult because of the potential for incorrect assembly of subunits. The IR-A and IR-B isoforms of the insulin receptor are made by alternative splicing of exon 11 in the insulin receptor gene and are expressed in a tissue-specific manner (Moller et al., 1989; Mosthaf et al., 1990; Goldstein and Dudley, 1990). Although this is not a problem with 7TM receptors, there are cases in which alternative splicing of messenger ribonucleic acid (mRNA) from a gene results in receptor isoforms for dopamine receptors (Giros et al., 1989; Monsma et al., 1989; Dal Toso et al., 1989) and for rhodopsin (Tanabe et al., 1992; Fong et al., 1992b; Sugimoto et al., 1993). In chromaffin cells, alternative splicing of the mRNA from the single gene encoding for the prostaglandin EP_3 receptor yields four receptor isoforms that differ only in their C-terminal tails. These differences determine differences in G-protein coupling (Namba et al., 1993). Alternate splicing is responsible for the different isoforms of receptors such as the GHRH receptor (Mayo, 1992), dopamine D_{2A} and D_{2B} receptors (Dal Toso et al., 1989), and the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor (Spengler et al., 1993). Although there are five cloned somatostatin receptor types, a further degree of diversification has been observed with the alternative splicing to produce mSSTR2A and mSSTR2B (i.e., Reisine et al., 1993). The two isoforms differ in the cytoplasmic C-terminus (Vanetti et al., 1992) and show different coupling efficiency to adenylate cyclase and propensity to desensitize (Vanetti et al., 1993b).

Posttranslational changes in dopamine D_2 receptors have been reported to account for differences in ligand affinity (Giros et al., 1989; Monsma et al., 1989). There are several biochemical modifications of receptor proteins that can be made including glycosylation, palmitoylation, terminal amino acid acylation, amino acid cyclization, carboxy-terminal amidation, sulfation (tyrosine residues), phosphorylation, hydroxylation, and methylation. Some of these are more important than others for various receptors. A key modification that may be important for receptor systems co-expressed with G-proteins (vide infra), is prenylation, both in terms of targeting to the membrane and the signaling from the receptor system (Casey, 1995). Receptor glycosylation can cause differences in the size of receptors. For example, a marked tissue difference in glycosylation has been noted for the angiotensin type 2 receptor in human myometrium, murine fibroblasts and rat PC-12 cells (Servant et al., 1994) and for opioid receptors in various tissues (Liu-Chen et al., 1993). Some modifications, such as palmitoylation, can be affected by external stimuli (Bonatti et al., 1989; Omary and Trowbridge, 1981; Alvarez et al., 1990). For 7TM receptors, palmitoylation may be particularly important because it reg-

ulates signal transduction both from receptors and the G-proteins with which they interact (Bouvier et al., 1995).

Clearly, a great deal of artifactual data can result from incomplete or incorrect expression of receptor protein from genetic material. This is an uncontrolled variable in receptor expression. For the purposes of this review, it will be assumed that the pharmacological effects observed are not the result of posttranslational modification differences, but, rather, that they reflect the behavior of natural receptors.

With the first cloning of a cDNA encoding a G-protein coupled receptor (retinal photon receptor and rhodopsin) (Nathans and Hogness, 1983) and the following description of the cDNA sequence for the β_2-adrenergic receptor (Dixon et al., 1986) has come a cornucopiea of expressed animal and human 7TM receptors. Table 2 shows a partial list of cloned 7TM receptors. This table is meant as a source of information regarding the behavior of different receptors in different expression systems. It should be noted that this table is not meant to be a complete listing of all cloned receptors, nor should it be used to ascribe temporal organization with respect to when the individual receptors were first expressed. Also, cloned genes are not included, only studies in which the gene is expressed in a cellular system and the binding and/or function of ligands on the expressed receptor is studied.

With this new technology has come the potential for new concerns in receptor pharmacology that stem from the unique nature of 7TM receptors as recognition and transduction units. These new concerns stem from the expectation that receptor activity will be immune to the removal of a receptor from its native environment and the expression of that receptor into a foreign one. Hopefully, this review will outline the limits for this expectation and some strategies for recognizing when observed effects reflect innate ligand receptor activity and when it might reflect activity modified by receptor environment. This involves the identification of system-dependent rather than solely receptor-type-dependent potency of ligands. Clearly, it would be advantageous to recognize the latter situation in terms of the subsequent expectation of drug activity in a therapeutic environment.

IV. Definitions

As a preface to the discussion, it is useful to define some terms to be used throughout this review. Drugs will be assumed to have two basic properties, *affinity* and *efficacy*. The first term relates to how well the drug binds to the receptor (as defined by the equilibrium dissociation constant of the drug-receptor complex). The second term relates to what happens to the receptor system as a result of the drug binding. The effects may promote physiological response, in which case the drug demonstrates *positive efficacy* and therefore is defined as an *agonist*. Conversely, the drug may do nothing to the receptor but bind to it and by its presence preclude activation of the receptor by an agonist. This would make it a *neutral antagonist* with *zero efficacy*. Recent data compels yet another scenario in which receptor systems produce measurable physiological response in the absence of agonist (vide infra). Such receptor systems are defined as being *constitutively active* and may be used to discover drugs that destabilize active receptor complexes. Such drugs are referred to as *inverse agonists* and have *negative efficacy*. It should be noted that in the absence of constitutive receptor activity, neutral antagonists and inverse agonists behave in an identical manner. However, it should not be assumed that they are pharmacologically the same because important differences in the receptor properties of neutral antagonists and inverse agonists may be very relevant to the therapeutic use of these drugs and the classification of drug receptors with them in heterologous expression systems. At this point, it is useful to suspend the common usage of the term efficacy as the property of a drug that promotes positive physiological response and consider efficacy to be the *property of a drug that modifies subsequent interaction of the 7TM receptor with other membrane proteins* (Kenakin, 1994).

V. 7TM Receptor Behavior

The behavior of 7TM receptors can be divided into two components namely, *intrinsic* and *interactive*. Intrinsic behavior refers to the basic properties of receptor proteins to exist in multiple conformational states and the effects of those states on observed drug activity. The interactive behavior relates to the result of receptor and G-protein interaction on the quality and quantity of drug response. It is worth discussing these separately.

A. Intrinsic Receptor Behavior

There is considerable circumstantial evidence to suggest that 7TM receptors can exist in 'active' and 'inactive' conformational states with respect to the fruitful interaction with G-proteins. It is useful at this point to consider the analogy with 'two-state' theory for ion channels (Katz and Thesleff, 1957) as applied to receptors (Colquhoun 1973; Karlin, 1967; Thron, 1973; Robertson et al., 1994). This hypothesis describes a population of receptors that can exist in a so-called 'inactive' state T (following the convention for ion channels) and an 'active' state R, the relative proportions of which are defined by an allosteric constant L (where $L = [T]/[R]$). A drug [A] binds to the two conformations T and R where the equilibrium dissociation constants of the resulting complexes are K_{AT} and K_{AR} respectively:

$$\begin{array}{ccc} & L & \\ T & \rightleftharpoons & R \\ K_{AT} \downarrow\uparrow & & \downarrow\uparrow K_{AR} \\ A\cdot T & & A\cdot R \end{array} \qquad [1]$$

TABLE 2
Cloned and expressed receptors

Receptor	Species	System	Reference(s)
Adenosine			
A_1	rat	A9-L + CHO	Mahan et al., 1991
	human	CHO	Libert et al., 1992
	canine	CHO	Libert et al., 1991
	rat	*Xenopus* oocytes	Yakel et al., 1993
A_{2b}	human	CHO-K1	Pierce et al., 1992
	sheep	COS-1/CHO K1	Linden et al., 1993
A_3	human	CHO	Salvatore et al., 1993
A_3			
Adrenergic			
α_1	hamster	COS-7	Cotecchia et al., 1988
α_1	bovine	COS-7	Schwinn et al., 1990
α_1	human	COS-7	Regan et al., 1988
α_2		CHO	Fraser et al., 1989; Lomasney et al., 1990
	mouse	COS-7	Link et al., 1992; Chruscinski et al., 1992
		Xenopus oocytes	Kobilka et al., 1987
	fish	COS-7	Svensson et al., 1993
	mouse		Link et al., 1992
α_{2C}	rat	CHO/COS-7	Voigt et al., 1991a
	human	COS-7	Link et al., 1992
α_{2A}	human	COS 1	Lanier et al., 1991
	rat	NIH 3T3	Duzic et al., 1992
α_{2B}, α_{2C}, α_{2D}	rat	COS	Zeng et al., 1990
α_{2B}	human	Ltk cells	Weinshank et al., 1990
	rat	COS 1	Lanier et al., 1991
α_{2D}	human	*Xenopus* oocytes	Kobilka et al., 1987
α_{2A}	porcine	COS-M6	Guyer et al., 1990
	rat	COS	Zeng et al., 1990
α_{2B}	human	SK-N-MC	Esbenshade et al., 1995a
α_{1D}	rat	L cells	Machida et al., 1990
β_1	human	*Xenopus* oocytes	Frielle et al., 1987
	mouse	COS-7/L-cells	Cohen et al., 1993
		sf9	Ravet et al., 1993
	human	CHO	Suzuki et al., 1991
β_1/β_2	mouse	Y-1	Allen et al., 1988
β_2	human	*Escherichia coli*	Marullo et al., 1988
	mouse	CHO	Nahmias et al., 1991
β_3	rat	CHO	Granneman et al., 1991
	human	CHO cells	Emorine et al., 1989
Calcium	bovine	*Xenopus* oocytes	Racke et al., 1993
Dopamine			
D_1	human		
	rat	COS-7	Zhou et al., 1990
	human	COS-7	Sunahara et al., 1991a
	rhesus	C_6 cells	Machida et al., 1990
	rat	COS-7	Tiberi et al., 1991
D_{1B}	human	COS-7	Stormann et al., 1990
D_2	mouse	COS-7	Montmayeur et al., 1991
	rat	COS-7	Chio et al., 1990
		CHO-6, DUK 25	
	rat	mouse fibroblasts	Bunzow et al., 1988
	human	CHO	Sokoloff et al., 1992
D_2/D_3	rat	LZR1, Ltk 59	Castro and Strange, 1993
D_2/D_3	rat	CHO	Sokoloff et al., 1990
D_3	human	COS-7	Van Tol et al., 1991
D_4	human	COS-7	Sunahara et al., 1991b
D_5			
Histamine			
H_1	bovine	COS-7	Yamashita et al., 1991
H_2	rat	CHO	Traiffort et al., 1992
	canine	L-cells	Gantz et al., 1991
	human	Colo-320	Gantz et al., 1991

TABLE 2
Continued

Receptor	Species	System	Reference(s)
Muscarinic	porcine	*Xenopus* oocytes	Kubo et al., 1986
muscarinic			Akiba et al., 1988
	porcine	CHO	Peralta et al., 1987
muscarinic	drosoph.	Y-1 cells	Shapiro et al., 1989
muscarinic	mouse	Y-1, L-cells	Shapiro et al., 1988
m1	human	CHO-K1	Buckley et al., 1989
	human	HEK	Peralta et al., 1987
m1, m2	human	CHO-K1	Buckley et al., 1989
m2	human	CHO-K1	Buckley et al., 1989
m3		CHO	Tobin et al., 1992
	chicken	Y1/CHO	Tietje and Nathanson, 1991
m4	human	CHO-K1	Buckley et al., 1989
	human/rat	COS-7	Bonner et al., 1988
m5		CHO-K1	Buckley et al., 1989
Opioid	mouse	PC-12	Raynor et al., 1994a
κ		COS-1	Yasuda et al., 1993
	human	COS-1	Zhu et al., 1995
	rat	COS-7/*Xenopus* oocytes	Minami et al., 1993
		COS-7	Nishi et al., 1993
			Li et al., 1993
	mouse	CHO-DGH4	Raynor et al., 1994a
δ	human	CHO	Evans et al., 1992
		COS	Kieffer et al., 1992
	rat	COS-7	Raynor et al., 1994b
μ		COS-7	Chen et al., 1993
		COS-7	Bunzow et al., 1995
	human	COS-7	Knapp et al., 1994
δ	mouse	COS-1	Yasuda et al., 1993
	human	COS-7	Raynor et al., 1994b
μ			
Peptides	human	COS-7	Tsuzuki et al., 1994
Angiotensin type 2	mouse	COS-7	Nakajima et al., 1993
	rat	COS-7	Kambayashi et al., 1993
Bradykinin	rat	*Xenopus* oocytes	McEachern et al., 1991
B_2	human	COS-7	Hess et al., 1992
Calcitonin	human		Gorn et al., 1992; Moore et al., 1992
	rat		Sexton et al., 1993; Albrandt et al., 1993
	pig	COS	Lin et al., 1991
Cholecystokin	human	COS	Ulrich et al., 1993
A	rat	*Xenopus* oocytes	Wank et al., 1992
	human	COS-7	Miyake et al., 1994
B			Lee et al., 1993
	porcine	COS-7	Loosfelt et al., 1989
Choriogonadotropin	rat	COS-7	Chang et al., 1993
Corticotropin releasing factor	human	COS-7	Webb et al., 1995
$Endothelin_B$	canine	COS-7	Kopin et al., 1992
Gastrin	rat	BHK	Jelinek et al., 1993
Glucagon	human	COS-7	Kakar et al., 1992
Gonadotropin releasing hormone	rat	HEK 293	Mayo, 1992
Growth hormone releasing hormone	rat	HEK 292	McFarland et al., 1989
Lutropin/luteinizing hormone	mouse	L cells	Gudermann et al., 1993a
	rat	293	Krause et al., 1992
Neuropeptide Y	human	Baculovirus	Aharony et al., 1993
$Neurotensin_A$	opossum	COS-7	Juppner et al., 1991
Parathyroid hormone	rat	COS	Abou-Samra et al., 1992
	rat	COS	Ishihara et al., 1992
Secretin	human	HEK 293	Patel et al., 1995

TABLE 2
Continued

Receptor	Species	System	Reference(s)
Somatostatin	mouse/human	CHO	Rens-Domiano et al., 1992
R1	rat	COS-7	Li et al., 1992b
	human	CHO	Yamada et al., 1992a
R2	mouse	CHO	Yamada et al., 1992a
R3	rat	COS	Meyerhof et al., 1992
	human	COS-1	Yamada et al., 1992b
	mouse	CHO	Yasuda et al., 1992
R4	human	COS-7	Demchysyn et al., 1993
	human	COS-1	Rohrer et al., 1993
	mouse	COS-1DM	Bruno et al., 1992
R4, R5	human	CHO-K1/COS-1	Raynor et al., 1993
R5	human	CHO-K1	O'Carroll et al., 1994
	human	COS-7	Panetta et al., 1994
Substance P	murine	*Xenopus* oocytes	Sundelin et al., 1992
	rat	COS	Yokota et al., 1989
	human	COS-7	Takeda et al., 1991
TSH	human	COS-7	Misrahi et al., 1990
Thyrotropin	canine	COS	Parmentier et al., 1989
	rat	CHO-K1	Endo et al., 1995
VIP	rat	COP	Ishihara et al., 1992
	human	COS-6	Sreedham et al., 1991
5-HT			
1	rat	HEK 293	Voigt et al., 1991b
1A	rat	Ltk^-	Albert et al., 1990
	human	monkey kidney	Fargin et al., 1988
		NIH 3T3	Varrault et al., 1992
1B	rat	Y-1	Adham et al., 1993
	human	sf9	Ng et al., 1993
		HeLa	Hamblin et al., 1992
	mouse	NIH 3T3	Marotoeaux et al., 1992
1C	human	*Xenopus* oocytes	Julius et al., 1988
	mouse	*Xenopus* oocytes	Yu et al., 1991
1D	canine	COS-7	Maenhaut et al., 1991
	human	CHO-K1	Hamblin and Metcalf, 1991
1E	human	murine L cells	Guderman et al., 1993b
2	rat	(mammalian)	Pritchett et al., 1988
2B	human	AV12-664	Kursar et al., 1994
3	mouse	COS-1/*Xenopus* oocytes	Maricq et al., 1991
5A, 5B	mouse	COS-7	Matthes et al., 1993
5A	human	Cos M6	Rees et al., 1994
(S12)	human	Ltk	Levy et al., 1992
7	rat	COS-7, HEK 293	Shen et al., 1993
GP2-7	guinea p	CHO-K1	Tsou et al., 1994

The important thing to note from this scheme is that unless A has *identical* affinities for T and R, the presence of A will *alter* the relative proportions of T and R, i.e., drug A plays an active role in the equilibrium and is not a mere observer. Under these circumstances, the fraction of receptors in the activated form in the presence of any given concentration of ligand (normalized to $c = [A]/K_{AR}$) is given by:

$$\rho = \frac{1}{1 + L[(1 + Mc)/(1 + c)]} \qquad [2]$$

where M is the ratio of equilibrium dissociation constants of A for the two receptor states ($M = K_{AR}/K_{AT}$). Thus, a measure of the ability of A to alter the equilibrium is denoted by M, and a correlate to drug efficacy in this type of system can be given by (Colquhoun, 1973);

$$\epsilon = \frac{K_{AT}}{K_{AR}} - 1 = \frac{1}{M} - 1 \qquad [3]$$

There are two features of this type of system to note. The first relates to the drug constant M. There is no a priori reason to assume that a ligand will promote only receptor activation; in fact, it is equally possible that a ligand will destabilize activated receptor formation (i.e., have a selectively higher affinity for the inactivated receptor T). In a system where there are few activated receptors in the absence of ligand, drugs with selective affinities for the inactivated state will have little effect,

i.e., it is not possible to turn off a system that already is turned off. However, if there is appreciable R present because of a favorable L, then a ligand with selective affinity for T over R will *decrease* spontaneous receptor activity. Such a destabilizing property of the ligand would not be detectable in the absence of constitutive receptor activity.

The maximal receptor activation of receptors by a saturating concentration of ligand is given by:

$$\rho_{max} = \frac{1}{1 + L \cdot M} \quad [4]$$

and it can be seen from equation 4 that it is the product of L and M that determines the observed drug effect. Therefore, for a destabilizing ligand (also called an inverse agonist), (vide infra) where $M > 1$, no appreciable effect will be observed if the magnitude of the allosteric constant L is very small (i.e., $LM \rightarrow 0$).

The second idea relates to the allosteric constant L. The fraction of receptors in the activated state in the absence of ligand A is given by:

$$\rho = \frac{1}{1 + L} \quad [5]$$

Theoretically, it is possible to have a system where *all* of the receptors are in the active state (to produce a 'constitutively' fully activated receptor system). Under these circumstances, an agonist would produce no measurable response, as the system will already reside at the maximal asymptote for response. Also, a system in which the constant L is large, even an extremely efficacious ligand with powerfully selective affinity for only the activated state R, would not be able to effectively change the relative quantities of T and R. Thus, the *system* could impose the dominant regulation on drug activity.

The intrinsic behavior of 7TM receptors is germinal to the overall behavior of these receptors in expression systems. However, another property is equally important and sets these receptors apart from ion channels, namely the ability to translocate within the membrane and interact with other membrane bound proteins.

B. Interactive Behavior: Cellular Host Effects

There are tissue-specific effects on receptors that modify observed drug activity. These effects can be quite striking, as in the case of 5-HT receptor-mediated responses in brain regions. Whereas the agonist activities of serotonin and a range of other agonists is comparable in mouse hippocampus and cortical neurons, methysergide and metergoline are nearly full agonists in hippocampal neurons and complete antagonists in cortical neurons (Dumius et al., 1988).

Just as different regions of organs such as the brain may have different cellular hosts for 7TM receptors, the transfection of these receptors into different expression systems may cause differences in receptor behavior. Surrogate cell lines for the expression of receptors generally are chosen for technical reasons, i.e., robust expression levels, etc. There is a considerable body of evidence to show that many 7TM receptors appear to function normally when introduced into these systems. Antagonist profiles can be remarkably similar for a given cloned receptor in different cell lines. However, such positive evidence is less revealing than negative evidence, i.e., it never can be proven that a receptor behaves in a physiologically normal fashion in a surrogate cell line, only when it does not. Thus, the weight of confirming evidence may only belie the fact that drugs that would show the differences have not yet been tested.

Cellular hosts can have long-term effects on 7TM receptors. For example, while the human β_3-adrenergic receptor desensitizes to isoproterenol in SK-N-MC cells and 293 cells, it does not do so in Chinese hamster ovary (CHO) cells. Similarly, the rat β_3-adrenergic receptors that does not desensitize in rat adipocytes does so in 293 cells (Chaudry and Granneman, 1994). Similar differences for β_3-adrenergic receptor desensitization have been found in L and Chinese hamster fibroblasts cells (Nantel et al., 1995).

The most obvious cases of deficient host effects are for agonists because this activity requires the coupling of the activated receptor to a G-protein. If the appropriate G-protein is not present in the surrogate cell, or the stoichiometry of the receptors and G-proteins is aberrant, then different activity may result. The effects of this phenomenon on high affinity agonist binding will be dealt with separately. As well as host cell deficiencies, receptors may be expressed in cells containing components they do not normally encounter in native tissues, or that they do encounter but with different stoichiometries. A striking case of functional reversal was demonstrated by Duzic and Lanier (1992) who transfected the α_{2B}-adrenergic receptor into three cell lines. In DDT$_1$-MF2 and NIH 3T3 cells, the α_{2B}-adrenergic receptor agonist epinephrine produces a concentration-dependent inhibition of cyclic adenosine monophosphate (cAMP). Surprisingly, an *increase* in cAMP is mediated by this agonist in PC-12 cells (fig. 1).

Cellular hosts can affect parameters fundamentally thought to reflect receptor properties. For example, the potency ratios for porcine and human calcitonin, human calcitonin gene related peptide (CGRP), and rat amylin can be shown to vary dramatically for activation of porcine calcitonin receptors transfected in either CHO or COS cells (Christmanson et al., 1994). Whereas absolute potencies may vary between systems, relative potencies should not vary unless different G-proteins are activated selectively by the activated receptors in the two hosts. This effect of 'stimulus trafficking' by agonists is discussed more fully later.

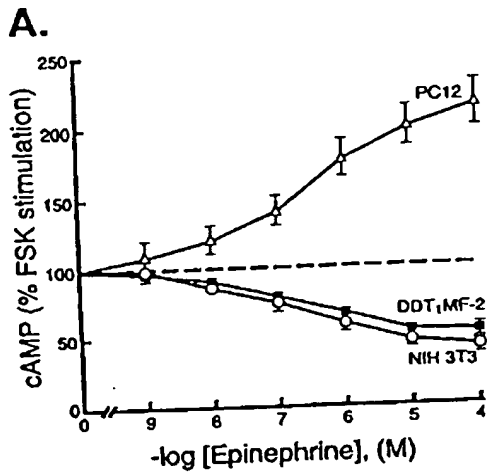


FIG. 1. (A) Effects of epinephrine on α_{2B}-adrenergic receptors transfected into three different cell lines. Ordinates: levels of cytosolic cAMP. Abscissae: Logarithms of molar concentrations of epinephrine. With permission from the American Society for Pharmacology and Experimental Therapeutics (ASPET) and from Duzic and Lanier (1992).

Different host cells may present receptors with different arrays of G-proteins. For example, expression of α_{1B}- and α_{1D}-adrenergic receptors in COS-1 and CHO cells leads to two different couplings. In COS-1 cells, coupling is to a pertussis toxin-insensitive G-protein that leads to phosphosinositol (PI) hydrolysis and increased cAMP and a pertussis-insensitive G-protein to an L-type calcium channel to stimulate phospholipase A. In CHO cells, the coupling is via a pertussis-insensitive G-protein to increase PI hydrolysis (Perez et al., 1993). Another effect of the host cell system on responses of transfected receptors was reported for the chicken muscarinic m2 receptor which, when expressed in Y-1 cells, inhibited adenylate cyclase, but when expressed in CHO cells, inhibited adenylate cyclase and stimulated phosphoinositide metabolism (Tietje and Nathanson, 1991). A striking effect was obtained in cells transfected with high levels of 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors when antagonists such as yohimbine and dihydroergotamine produced agonist effects (Adham et al., 1993). Table 3 shows other examples of receptors in various cellular hosts that demonstrate differences in effector coupling.

In addition to effects on receptor/G-protein coupling, there are other tiers of interaction in cellular signaling. For example, there is evidence that 7TM receptors can interact with each other, as in the case of the abolition of natural 5-HT_1-like receptor effects in CHO cells by transfection and activation of 5-HT_{2C} receptors (Berg et al., 1994).

In general, it can be said that the automatic assumption of immutable receptor behavior irrespective of cellular host is not supported by data. The proclivity of receptors to interact with many G-proteins spontaneously (vide infra) raises the specter of the introduction of an uncontrolled variable in expression studies that may transfer to the observed activity of drugs.

TABLE 3

Coupling of the same receptor to different cytosolic cascades in different systems

Receptor	Systems	Reference(s)
Dopamine D_2	Rat striatum Rat anterior pituitary Pituitary GH_4C_1 cells Ltk$^-$ fibroblasts	Meller et al., 1992 Vallar et al., 1990
α_{2B}-Adrenergic	NIH 3T3 cells	Duciz and Lanier, 1992
Serotonin 5-HT_{1C}	PC12 cells DDT_1 MF-2 cells Natural systems Syrian hamster tumor cells	Lucaites et al., 1992
α_{1B}-Adrenergic α_{1D}-Adrenergic	COS-1 cells, CHO cells	Perez et al., 1993
Muscarinic m2	Y-1, CHO	Tietje and Nathanson, 1991

C. Evidence for Spontaneous Receptor/G-Protein Coupling

One of the major characteristics of 7TM receptors is the fact that they have different recognition domains for ligands and G-proteins. This latter property confers the ability of the receptor, when in the active state, to couple to and activate G-proteins. Sequence similarity dendograms have shown that 7TM receptor evolution can be traced at two sites, namely the ligand *and* G-protein binding sites (Donnelly et al., 1994). There is now a large body of evidence to show that many receptors can spontaneously couple to G-proteins in the absence of agonists. For example, solubilized CGRP receptors from rat cerebellum were shown to bind ^{125}I-CGRP with high affinity. Treatment of the solubilized receptor supernatant with $G_{\alpha s}$ antiserum caused immunoprecipitation of the G_s with a concomitant loss in receptor binding upon centrifugation (Chatterjee et al., 1993). The most straightforward explanation for these data is that the loss in receptor binding represented G_s protein-receptor complexes that were present in the supernatant spontaneously in the absence of CGRP.

There are numerous systems in which receptors can be purified as complexes with G-proteins. For example, solubilized D_2 receptors from bovine striatum copurify with G_i and G_o (Elazar et al., 1989). The D_2-dopamine receptor of the bovine anterior pituitary copurifies with affinity chromatography with a pertussis toxin-sensitive G-protein (Senogles et al., 1987).

Another line of evidence to show receptor precoupling comes from receptor kinetic studies. For example, the biphasic kinetics of N-formyl peptide receptor binding are amenable to explanation by the proposal that the receptor population exists as a mixture of precoupled

and uncoupled states (Fay et al., 1991; Posner et al., 1994). Similar data have been obtained for the α_2-adrenergic receptor (Neubig et al., 1988).

In general, there are a great deal of data to suggest that most 7TM receptors spontaneously couple to G-proteins in the absence of agonist. Table 4 gives some examples of these systems. The assumption that the spontaneous association of receptor with G-protein involves the receptor in the 'activated' form is supported by evidence that shows spontaneous receptor/G-protein coupling is associated with the production of physiological response, i.e., elevation of cAMP (Samama et al., 1993; phosphoinositide turnover; Senogles et al., 1990) and guanine nucleotide G-protein exchange (Costa and Herz, 1989; Freissmuth et al., 1991). For example, figure 2 shows the activation of $G_{o,i}$-protein by adenosine A_1 receptors. This activity can be increased by the agonist N^6-(phenylisopropyl)-adenosine and reduced by the inverse agonist xanthine amine congener (XAC). Of relevance to this discussion is the fact that there is measurable spontaneous activation of the G-protein in the absence of agonists.

D. Receptor/G-Protein Promiscuity

It is now well known that many 7TM receptors are able to activate multiple biochemical cascades. This also can be shown in receptor expression systems: table 5 shows examples of multiple signaling from single receptors when they are expressed in surrogate cell lines. Multiple signaling can be the result of multiple receptor coupling at the membrane level or it can be the result of the activation of multiple internal biochemical cascades. For example, bradykinin receptors in cultured rat mesangial cells depress cAMP through a phospholipase C pathway (i.e., production of diacyglycerol from stimula-

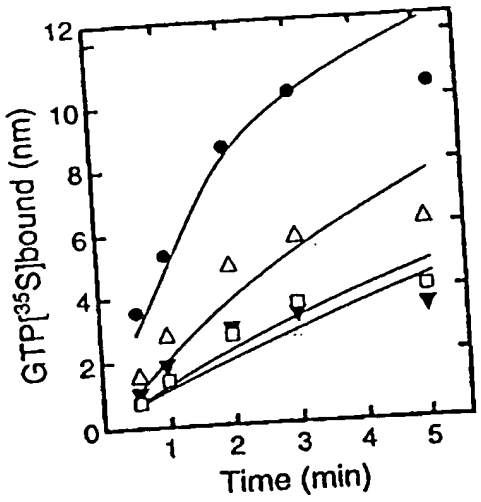


FIG. 2. Time course of GTP[^{35}S] binding to bovine brain $G_{o,i}$. Spontaneous G-protein activity in buffer alone (□), with adenosine A_1-receptor(△), adenosine receptor agonist R(−)-N^6-(2-phenylisopropyl)adenosine (●) and inverse agonist [^{3}H]xanthine amine congener (inverted filled triangles). With permission from the Biochemical Society and Portland Press and from Freissmuth et al., 1991.

TABLE 4
Evidence receptor precoupling to G-proteins

Receptor	System	Reference(s)
Opioid	NG108-15 cells	Costa et al., 1990
	Rat brain	Demoliou-Mason and Barnard, 1986
		Wong et al., 1989
		Georgoussi et al., 1995
		Li et al., 1992a
Dopamine D_1	Canine/bovine striatum	Niznik et al., 1986
Dopamine D_2	Bovine anterior pituitary	Wreggett and De Lean, 1984
		Senogles et al., 1987
		Senogles et al., 1990
	Reconstitution	Senogles et al., 1990
CGRP	Rat cerebellum	Chatterjee et al., 1993
Somatostatin	Rat brain	Law et al., 1991
Purinergic P_{2Y}	Turkey eryth.	Jeffs et al., 1991
Muscarinic	Rat cerebral cortex	Baron et al., 1985
		Matesic et al., 1989
β-Adrenergic	Cardiac membrane	Nerme et al., 1986
α_2-Adrenergic	Calf cerebral cortex	Sladeczek et al., 1984
	Rat brain	Matsui et al., 1985
	Human platelets	Neubig et al., 1988
	bovine aorta	Jagadeesh et al., 1990
Adenosine A_1	Bovine corebral cortex	Leung and Green, 1989
Vasopressin		Fitzgerald et al., 1986

TABLE 5
Receptors coupled to multiple cytosolic cascades in cells

Receptor	System	Reference(s)
Muscarinic m3	IMR-32 cells	Pinkas-Kramarski et al., 1990
Muscarinic m1	CHO cells	Gurwitz et al., 1994
Muscarinic m2	CHO cells	Tietje and Nathanson, 1991
α_2-adrenergic	CHO cells	Fraser et al., 1989
		Eason et al., 1992
	CHO-K1 cells	Gerhardt and Neubig, 1991
PTH	COS cells	Abou-Samra et al., 1992
α_{1B}-Adrenergic	COS-1 cells	Perez et al., 1993
α_{1D}-Adrenergic	COS cells	Van Sande et al., 1993
Human 5-HT_{1D}		
Dog 5-HT_{1D}	Y1 Kin-8 cells	Van Sande et al., 1993
Human bradykinin B_2	CHO cells	Hess et al., 1994
Human calcitonin	BHK cells	Moore et al., 1992
Luteinizing H	Xenopus oocytes	Gudermann et al., 1993a
Rat endothelin A	Cardiac myocytes	Hilal-Danda et al., 1994
Human secretin	HEK-293 cells	Patel et al., 1995

tion of protein kinase C (PKC) inhibits stimulated cAMP production; Bascands et al., 1993). However, there are mechanisms whereby a single receptor can activate more than one biochemical pathway at the membrane level. For example, a single G-protein interaction can activate more than one biochemical cascade as in the activation of adenylate cyclase and phospholipase C by 5-HT_{1A} receptors (Fargin et al., 1991). Similarly, it has been shown that heterotrimeric G-proteins containing $G_{\alpha i3}$ can regulate multiple effector enzymes in the same cell (Hunt et al., 1994). As well as effector activation by α-subunits of G-proteins, it is now clear that the $\beta\gamma$ counterparts can directly activate effectors (Logothetis et al., 1987; Jelsma and Axelrod, 1987; Whiteway et al., 1989; Birnbaumer, 1992; Tang and Gilman, 1992; Camps et al., 1992; Katz et al., 1992; Blank et al., 1992; Iniguez-Lluhu et al., 1993; Boyer et al., 1994; Muller and Lohse, 1995). These effectors include adenylate cyclase, phospholipase A_2, K^+ channels, phospholipase C, calcium channels and receptor kinases (Clapham and Neer, 1993). This adds another level of complexity into 7TM receptor/G-protein signaling because the presence or absence of counterpart effectors for $\beta\gamma$-dimers of receptor linked G-proteins will affect the type and magnitude of agonist response. For example, a natural cellular system for a given receptor may contain a dedicated G-protein which, upon agonist-receptor activation, yields an α-subunit that interacts with one effector and a $\beta\gamma$ dimer that activates another. The summation of the effector cascades produce the cellular response. If this receptor is transfected into another cell type that has the appropriate G-protein, it still will not produce the same response unless *both* effectors for the α and $\beta\gamma$-dimer subunits are present in the membrane as well.

While receptor signaling can yield pleiotropic responses in the cytosol, there also is considerable evidence to show that receptors demonstrate G-protein specificity. For example, adenosine A_1 and dopamine D_2 receptors transfected into HEK 293 cells activate G_i (Wong et al., 1992) but do not interact with α_q to activate PLC, even when this subunit is overexpressed (Conklin et al., 1993). Structure-activity selectivity was shown in this latter study when a three-amino-acid substitution switched receptor selectivity of $G_{\alpha q}$ to that of $G_{\alpha i}$ (Conklin et al., 1993). Furthermore, although the adenosine A_1 receptor and dopamine D_2 receptors are indistinguishable in activation of G_i, they did discriminate chimeras of α_q to α_i (Conklin et al., 1993). Similarly, 5-HT_{1A} receptors expressed in *Escherichia coli* form high affinity agonist complexes with several G-protein α-subunits but neglect to do so with others (See fig. 3A).

It is well known from recombinant and natural systems that there is cross-reactivity of receptors between many different G-proteins. For example, Haga and coworkers (1989) have shown that the muscarinic receptor forms high affinity complexes with acetylcholine equally well when reconstituted with G_o, G_i and G_n. It has been found that cloned human 5-HT_{1D} receptors expressed in CHO cells and that dog 5-HT_{1D} receptors expressed in Y1 Kin-8 cells can both stimulate and inhibit adenylate cyclase by concomitantly interacting with G_s and G_i proteins (Van Sande et al., 1993). The α_{2D}-adrenergic receptor expressed in S115 mouse mammary tumor cells inhibits adenylate cyclase via G_i *and* increases cAMP upon treatment of the cells with pertussis toxin, presumably via G_s (Jansson et al., 1994). Kinetic studies have been used to delineate receptor/G-protein promiscuity. For example, the interaction of the thyrotropin-releasing hormone receptor with $G_{q/11}$ and another unidentified G-protein was inferred from the observation of biphasic kinetics of [^{35}S]GTPγS binding (Brady et al., 1994). Some other examples of this promiscuity at the biochemical level are given in table 6A. This promiscuity carries over to transfected receptors in cellular expression systems (table 6B).

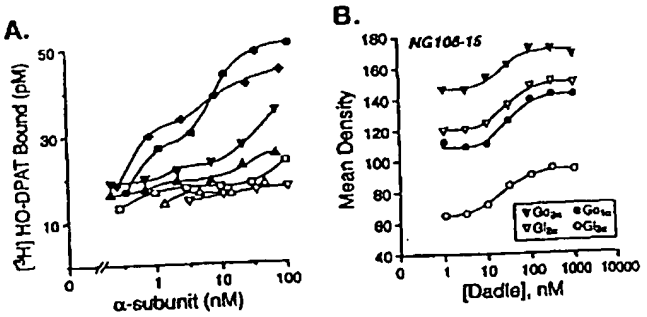


FIG. 3. Selectivity and promiscuity between receptors and G-proteins. (A) Interactions of 5-HT_1 receptors in *E. coli* membranes with various G-proteins. Formation of high affinity binding for [^{3}H]DPAT (ordinates) versus molar concentration of G-protein α-subunit (logarithmic scale). Data for $G_{\alpha i3}\beta\gamma$ (filled diamonds), bovine brain $G_{o,i}$ (filled circles), rat $G_{\alpha i2}\beta\gamma$ (inverted filled triangles), rat $G_{\alpha i1}\beta\gamma$ (filled triangles), and a lack of ternary complex formation with rat $G_{\alpha o}\beta\gamma$ (open squares), rat $G_{\alpha i1}\beta\gamma$ (open triangles) and rat $G_{\alpha s}\beta\gamma$ (open inverted triangles). With permission from the Journal of Biological Chemistry and from Bertin et al. (1992). (B) Radioactive labeling of complexes between opioid receptors, the opioid agonist DADLE and various G-protein α-subunits in NG108-15 cells. With permission from ASPET and from Prather et al. (1994).

There also are numerous examples of natural systems in which a single receptor activates more than one G-protein (see table 7). In NG108-15, neuroblastoma × glioma cells the opioid receptor agonist [D-Ala^2, D-Leu^5]enkephalin (DADLE) has been shown to form three complexes with G_o, G_{i2}, and G_{i3} (Roerig et al., 1992). Adenosine A_1 receptors from bovine brain have been shown to copurify with G_{i1}, G_{i2}, and G_o (Munshi et al., 1991). Similarly, the muscarinic receptor in cerebellar and cardiac ventricular membranes was shown to form complexes with both G_i and G_o when activated by the agonists carbachol, pilocarpine and McN A343 (Matesic et al., 1991). Solubilized D_2 receptors from bovine striatum copurify with G_i and G_o (Elazar et al., 1989) and, as shown by Senogles et al. (1990), purified D_2 receptors activate GTPase of G_{i1}, G_{i2}, and G_{i3}. In patch

TABLE 6

Receptor	Coupler	Reference
Known Cross-Reactivity Between Receptors and G-proteins		
Muscarinic	G_i, G_o	Florio and Sternweis, 1985
	G_i, G_o	Haga et al., 1986; Kurose et al., 1986
	G_i, G_o, G_n	Haga et al., 1988, 1989
	G_i, G_p	Ashkenazi et al., 1987
	G_i, G_s	Dittman et al., 1994
	G_i, G_s	Asano et al., 1984
β-Adrenergic	G_i, G_s	Marbach et al., 1988
	G_i, G_s	Rubinstein et al., 1991
	G_s, G_{i1}, G_{i3}	Cerione et al., 1986
α_2-Adrenergic	G_i, G_s	Kim and Neubig, 1987
	G_i, G_o	Milligan et al., 1991
	G_{i2}, G_{i3}	Gerhardt and Neubig, 1991
	G_{i2}, G_{i3}	Eason et al., 1994
	G_i, G_s	Fraser et al., 1989
	G_i, G_s	Roth and Chuang, 1987
Serotonin	G_i, G_k, G_p, G_s	Raymond et al., 1993
	$G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$	Asano et al., 1985
γ-Aminobutyric acid B	G_i, G_o	Ohara et al., 1988
Dopamine D_2	G_i, G_o	Kimura et al., 1995
	G_{i1}, G_{i2}, G_{i3}	Senogles et al., 1990
Opioid	G_o, G_{i2}	Offermanns et al., 1991
Adenosine	G_{i2}, G_{i3}, G_o	Roerig et al., 1992
	G_{i1}, G_{i2}, G_o	Munshi et al., 1991
Neuropeptide Y	G_s, G_{i1}, G_{i2}	Ewald et al., 1989
Bradykinin	G_s, G_{i1}, G_{i2}	Ewald et al., 1969
Somatostatin	$G_{\alpha i1}$, $G_{\alpha i3}$	Law et al., 1991
	$G_{\alpha i2}$, $G_{\alpha o}$	Luthin et al., 1993
Calcitonin	G_s, G_i, (G_p)	Chakraborthy et al., 1991
5-HT_{1A}	$G_{\alpha i2}$, $G_{\alpha i3}$	Gettys et al., 1994
Evidence of Receptor/G-Protein Promiscuity in Transfected Cellular Systems		
α_{2B}-Adrenergic	S115 cells	G_i (G_s)	Jansson et al., 1994
α_{2A}-Adrenergic	Rat 1 fibroblasts	G_{i2}, G_{i3}	Milligan et al., 1991
		G_{i2}, G_{o1}	Grassie and Milligan, 1995
α_{2A}-Adrenergic	CHO cells	G_i, G_s	Eason et al., 1994
	LLC-PK1-O	G_{i1}, G_{i2} G_{i3}, G_o	Okuma and Reisine, 1992
Muscarinic m4	HEK 293 cells	G_i, G_s	Dittman et al., 1994
5-HT_{1A}	CHO cells	$G_{\alpha i2}$ $G_{\alpha i3}$	Gettys et al., 1994
5-HT_{1E}	BS-C-1 cells	G_i, G_s	Adham et al., 1994b
5-HT_{1C}, TRH	*Xenopus*	G_o, G_q	Quick et al., 1994

clamp experiments, antisera to $G_{\alpha o}$ and $G_{\alpha i3}$ respectively reduced potassium currents caused by D_2 receptor activation (Lledo et al., 1992). When these types of interactions do occur, the concentrations of agonist producing the multiple ternary complex species usually are very similar, as in the formation of $G_{\alpha o2}$, $G_{\alpha o1}$, $G_{\alpha i2}$, and $G_{\alpha i3}$ complexes with DADLE and opioid receptors in NG108-15 cells (see fig. 3B) (Prather et al., 1994).

TABLE 7

Possible "naturally promiscuous" receptor systems

Tissue	Receptor	Reference(s)
Chick heart	Muscarinic	Agnarsson et al., 1988
		Brown and Brown, 1984
		Brown and Goldstein, 1986
		Tajima et al., 1987
Rat atrium	Muscarinic	Eglen et al., 1988
		Imai and Ohta, 1988
		Kenakin and Boselli, 1990a, b; 1991
Guinea pig atrium	Muscarinic	Eglen et al., 1988
		Imai and Ohta, 1988
Rat striatum	Muscarinic	Kelly et al., 1985
Rat medulla pons	Muscarinic	Birdsall et al., 1980
Neuroblastoma cells	Muscarinic	Bruni et al., 1985
7315c cells	Angiotensin	Crawford et al., 1992
Rat anterior pituitary	Angiotensin	Enjalbert et al., 1986
3T3 fibroblasts	Thrombin	Murayama and Ui, 1985
Hippocampus (rat/guinea pig)	Serotonin	De Vivo and Maayani, 1986
Rat hepatocytes	Glucagon	Wakelam et al., 1986
Rat phrenic nerve hemi-diaphragm	Adenosine	Silinsky et al., 1989
CHP212	CCK	Barrett et al., 1989
NG 108-15 cells	δ Opioid	Offermanns et al., 1991
Rat brain	Opioid	Wong et al., 1989
Bovine striatum	Dopamine D_2	Elazar et al., 1989
Rat anterior pituitary	Dopamine D_2	Lledo et al., 1992
αT3-1 cells	Gonadotrophin-releasing hormone	Shah and Milligan, 1994
LLC-PK_1 cells	Endothelin	Ozaki et al., 1994
Rat myometrium	Endothelin	Khac et al., 1994
Rat brain	Somatostatin	Murray-Whelan and Schlegel, 1992

Receptor/G-protein cross-reactivity also can be seen with studies of signal down-regulation: for example, in the immortalized gonadotroph cell line αT3-1 cells that express gonadotropin-releasing hormone (GnRH) receptor. Exposure to a GnRH receptor agonist results in substantial down-regulation of the α-subunits of G-proteins G_q and G_{11} (Shah and Milligan, 1994). These and other data in this study suggest that this receptor interacts functionally with both $G_{q\alpha}$ and $G_{11\alpha}$.

Although receptors can be promiscuous with respect to the G-proteins with which they interact, they can also be promiscuous with respect to cell cycle. For example, calcitonin receptors in LLC-PK1 cells interact with two G-proteins to activate the cAMP and PKC pathways via cholera toxin G_s and pertussis toxin sensitive G_i protein, respectively, to produce opposite biological responses (Chakraborthy et al., 1991). Interestingly, the primary activation of one pathway over the other was cell cycle-dependent (i.e., G2 versus S phase).

These data in general lead to a model for 7TM receptor systems that must contain interactive receptors, G-proteins and ligands. As a preface to discussion of a current model for such systems, it is useful to trace the history of receptor models in pharmacology.

VI. Receptor Models

The discussion of these ideas is considerably easier with comparison of experimental data with a receptor model. The first mathematical application of a receptor theory to data was made by Clark (1933, 1937), and invaluable modifications were made by Gaddum (1937, 1957) and Schild (1947a, b; 1949; 1957) among others (see Parascandola, 1986 for review). With the introduction of the concept of efficacy into drug-protein interaction (Ariens, 1954, 1964; Stephenson, 1956), the ideas relating to allosteric states of enzymes and ion channels (Monod et al., 1965; Koshland, 1960; Karlin, 1967; Katz and Thesleff, 1957; Thron, 1973; Colquhoun, 1973) and the idea that receptors can translocate within the membrane and interact with other membrane proteins (Cuatrecasas, 1974) have come the basic ternary complex model (DeLean et al., 1980). Subsequent receptor studies and the availability of new receptor test systems have caused the modification of this model into the extended ternary complex model (Leeb-Lundberg and Mathis, 1990; Samama et al., 1993). It should be recognized that there are numerous other models available to describe drug-receptor interaction (for review see MacKay, 1977; Kenakin, 1984).

Alternative models such as the operational model of receptors (Black and Leff, 1983) are not bound by mechanistic constraints and can be used to quantify drug activity in general terms. This is a particular advantage in functional analysis of drug-receptor interaction in which null methods are used to negate systems effects. Because this review is specifically concerned with 7TM receptors, the known biochemical mechanisms of these systems will be used for modeling purposes. A recently described statistically complete model of 7TM receptor/G-protein interaction, termed the cubic ternary complex model (Weiss et al., 1996a, b), will be used to describe ligand effects.

A. The Cubic Ternary Complex Model

In general, there are three classes of interaction in 7TM receptor systems; these are shown schematically in figure 4. Part I shows receptor activation as a equilibrium between the active (R_a) and inactive (R_i) receptor forms and their interaction with the ligand A. The affinity constant of the ligand for the inactive receptor is denoted K_A, and it is modified by a factor α that quantifies the difference in affinity the ligand has for the activated over the inactivated receptor. The allosteric constant describing the equilibrium between R_i and R_a is denoted K_{act}. The concept of microscopic reversibility

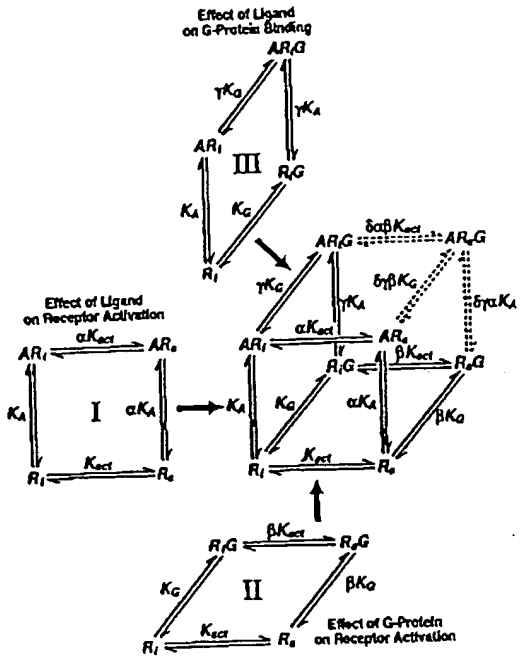


FIG. 4. Cubic ternary complex model for 7TM receptors. A. Three faces of the cube represent (I) the effect of ligand on receptor activation (A on R_i to R_a), (II) the interaction of receptors with G-protein (G on R_i and R_a) and (III) the added effect of ligand on receptor/G-protein interaction (A on R and G). (middle right) The completed cube with appropriate association constants mandatory by microreversibility.

(Wyman, 1975) sets the equilibrium association constants for the ligand bound receptor species to be αK_{act}.

The second class of interaction is the receptor behavior toward G- proteins (part II in fig. 4). Here it can be seen that, theoretically, both receptor forms can bind to G-protein; this is a deviation from the extended ternary complex model as described by Samama et al. (1993). Although there is no evidence that a stable complex between the inactivated receptor and G-proteins exists (species R_iG), all proteins have an unconditional association constant between them, albeit small; thermodynamically, a path must exist through this species for the system to be energetically correct. Thus, although K_G may be exceedingly small, the factor β can be large to favor coupling of the activated receptor over the inactivated receptor to G-protein. Again, microscopic reversibility sets the other equilibrium constant to βK_{act}.

Finally, the agonist effect on receptor/G-protein coupling is shown as part III in fig. 4. Here the presence of the agonist on the receptor produces a bias to receptor coupling by a factor γ. The full construction of the cube must interrelate these processes with the equilibrium constants shown in fig. 4 (described in detail in Weiss et al., 1996a, b). Clearly, the model is heuristic in that too many constants exist for useful modeling of data and ascription of chemical significance to ligand properties. However, the model is complete and is useful for describ-

ing and predicting receptor behaviors in different host systems. Moreover, the cubic ternary complex model subsumes many previous models of 7TM receptor systems; some of these are shown in table 8.

VII. Pharmacological Drug-Receptor Classification

As can be seen from the previous discussion, 7TM receptors can be thought of as societal proteins in membranes. They interact with other membrane proteins in promiscuous fashion and can carry on a signaling dialogue in the absence of agonists. When foreign ligands are thrust into this milieu, these systems are perturbed, and the manner in which the system adjusts to this perturbation yields measures of ligand-receptor activity that pharmacologists and medicinal chemists use to design drugs for therapeutic use. It is axiomatic that measures of drug activity must be independent of the systems from which they are obtained, and the usual methods to do this measure ligand *affinity* and ligand *efficacy* (Kenakin, 1984). These drug parameters should be unique for each receptor and thus transcend the measuring system to be predictive of activity in humans.

Theoretically, there are two approaches that can be taken to do this. One is the recreation of the physiological environment for the receptor of interest as the primary screening system for new drug entities. In view of the paucity of knowledge regarding the complete nature of these systems, this approach does not seem practical. A second approach, which strives to diminish the behavioral effects of receptors on drug activity and yield chemical constants of interaction, would appear to be more useful at present. The first step in this process is the recognition of when receptor behavior obscures and when it modifies observed drug activity.

The introduction of receptors into foreign host cells can produce artifacts with respect to the observed behavior of drugs. This can occur by mating the receptor with inappropriate membrane coupling proteins (heterologous match-making). Thus, the newly transfected receptor may be introduced to G-proteins not normally encountered. For example, the mouse 5-HT_{1C} receptor is known normally to couple to phospholipase C; however, when transfected into Syrian Hamster tumor cells, an unexpected inhibition of adenylate cyclase (blocked by pertussis toxin and thus related to receptor/G-protein interaction) was observed (Lucaites et al., 1992). Similar effects can be seen by varying the stoichiometry of 7TM receptors and G-proteins (vide infra). As a preface to the discussion of these issues, it is useful to consider the molecular nature of drug activity, namely the nature of affinity and efficacy. The two properties are native to molecules and therefore intimately related (i.e., see Colquhoun, 1987). The first step is to examine the conditions under which these properties can, if possible, be studied separately.

TABLE 8
7TM Receptor models

Model	Formulation	References
Classical	$A + R \rightleftharpoons AR$	Clark, 1933; 1937; Hill, 1909; Gaddum, 1937, 1957; Langley, 1878, 1909; Stephenson, 1956
Simple ternary complex	$A + R \rightleftharpoons AR$ $+$ G $\Updownarrow$ ARG	Ross, 1989; Bourne et al., 1990; Birnbaumer et al., 1990; MacKay, 1988, 1990; Mayo et al., 1989
Ternary complex	$R + A \rightleftharpoons AR$ $+ \quad +$ $G \quad G$ $\Updownarrow \quad \Updownarrow$ $RG + A \rightleftharpoons ARG$	Wreggett and DeLean, 1984; DeLean et al., 1980; Costa et al., 1992; Ehlert, 1985; Cuatrecasas, 1974; Jacobs and Cuatrecasas, 1976; Abramson et al., 1987; Boeynaems and Dumont, 1977; Neubig et al., 1988; Minton and Sokolovsky, 1990; Lee et al., 1986
Simple two-state	$R + A \rightleftharpoons AR$ $\Updownarrow$ AR^*	Karlin, 1967; Thron, 1973; Changeux et al., 1967; Katz and Thesleff, 1957; Kirschner and Stone, 1951; del Castillo and Katz, 1957
Full two-state	$R + A \rightleftharpoons AR$ $\Updownarrow \quad \Updownarrow$ $R^* + A \rightleftharpoons AR^*$	Iyengar et al., 1980; Birnbaumer et al., 1980; Colquhoun, 1973; Karlin, 1967; Podleski and Changeux, 1970; Heldenreich et al., 1980; Ross et al., 1977
Extended ternary	$R + A \rightleftharpoons AR$ $\Updownarrow \quad \Updownarrow$ $R^* + A \rightleftharpoons AR^*$ $+ \quad +$ $G \quad G$ $R^*G + A \rightleftharpoons AR^*G$	Leeb-Lundberg and Mathis, 1990; Samama et al., 1993; Lefkowitz et al., 1993

R*, activated receptor predisposed to G-protein coupling.

A. The Expectation of Zero Efficacy

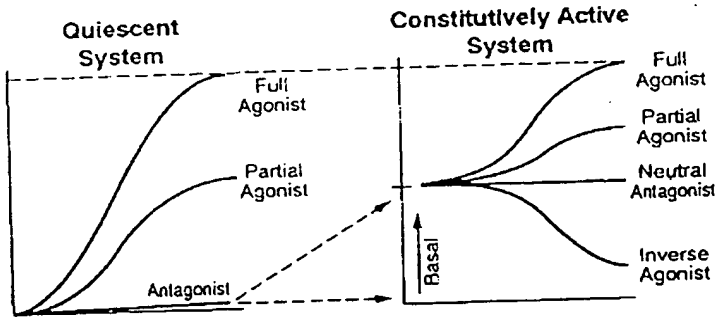
The efficacy of a ligand is usually observed as a change in the state of a receptor system in the presence of the ligand. By far, the predominance of experience with efficacious ligands has been in quiescent systems that demonstrate a ligand-induced physiological response. The first general idea to consider is the translation of ligand efficacy by the receptor system. Positive efficacy interacts with the intrinsic amplification stimulus- response mechanisms of the receptor system to yield an observable change of state. However, the sensitivity and power of these mechanisms can completely control what is observed. For example, the low β-adrenergic receptor efficacy of prenalterol can demonstrate full agonism in atria from thyroxine-treated guinea pig atria, partial

agonist activity in guinea pig left atria and *no* agonist activity in the extensor digitorum longus muscle of the guinea pig, where it acts as an antagonist (Kenakin and Beek, 1980; Kenakin, 1985a). Therefore, the lack of observation of an agonist response does not necessarily preclude the presence of ligand efficacy, only that the system was inadequate to make it observable (Kenakin, 1985a; Hoyer and Boddecke, 1993).

Considering efficacy as the property of a drug that, when it is bound to the receptor, modifies the interaction of that receptor with other membrane-bound proteins encompasses a larger potential than simply the production of cellular response. The cubic ternary complex model has a set of parameters that can be divided into those that are characteristic of the receptor system (K_G, K_{act}, β, [R], [G]) and those that are characteristic of the drug interacting with that system (K_A, α, γ, δ). If it is assumed that K_A is the chemical equilibrium dissociation constant of the complex between the inactive receptor and the ligand (i.e., a measure of true affinity), then the observed affinity of any ligand is given by (Weiss et al., 1996a):

$$K_{obs} = K_A \frac{1 + \alpha K_{act} + \gamma K_G[G] + \delta\alpha\gamma\beta K_G K_{act}[G]}{1 + K_{act} + K_G[G] + \beta K_G K_{act}[G]} \quad [6]$$

What can be seen from this equation is that, for K_{obs} to be equal to K_A, i.e., for simple affinity to be measured, then the condition that $\alpha = \gamma = \delta = 1$ must be true. If a drug has positive or negative efficacy (i.e., if either α, γ or δ are not equal to unity), then the observed affinity may be subject to systems conditions such as receptor/G-protein stoichiometry or level of spontaneous receptor activation. Figure 5 shows the observed affinity for a positive agonist (panel A), neutral antagonist (panel B), or inverse agonist (panel C) with changing level of receptor activation (K_{act}) and/or receptor expression level (G-protein level constant). As can be seen from this figure, the observed affinities of positive or negative agonists can vary with the system (i.e., cell type, receptor expression level). In general, positive agonism can increase the observed affinity of the ligand because the isomerization of the receptor to the active form (Colquhoun, 1985, 1987) and subsequent coupling to the G-protein creates a series of reactions that drives the binding of the agonist to the receptor beyond what would be dictated by the K_A (MacKay, 1987; 1988; 1990a, b; Leff and Harper, 1989; Kenakin et al., 1990). In contrast, the reverse is seen with inverse agonists. Because the higher affinity form of the receptor is the uncoupled



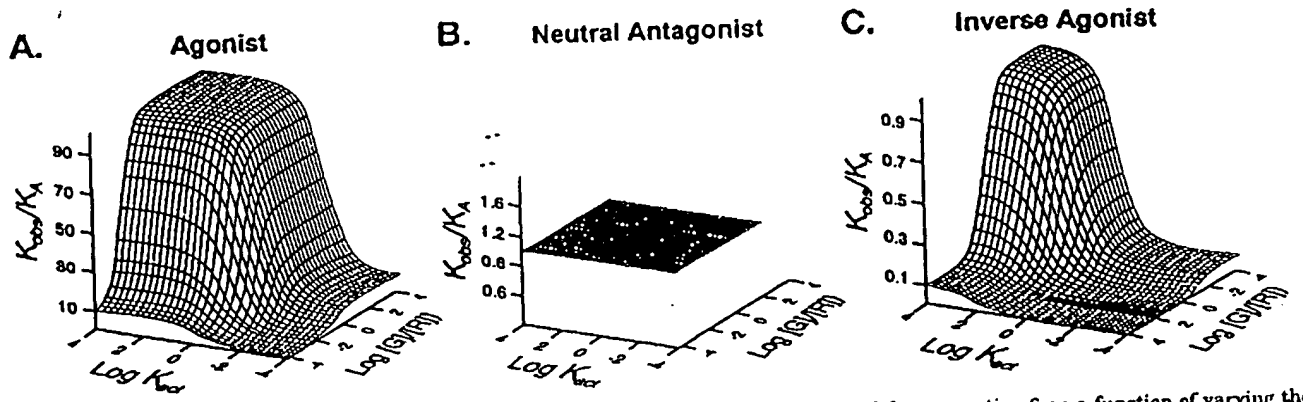


FIG. 5. Observed affinity of various types of ligands for 7TM receptors. K_{obs}/K_A calculated from equation 6 as a function of varying the ratio of receptors to G-proteins and varying setpoint levels of receptor activation (log K_{act}). Note how the systems parameters (K_{act} and [R]) have no effect on neutral antagonists (panel B) but increase the observed affinity for positive agonists (panel A) and decrease the observed affinity for inverse agonists (panel C).

free receptor, systems in which the receptor is precoupled to G-protein will demonstrate a lower observed affinity for the ligand. When uncoupling is complete, the observed affinity will equal K_A.

It can be seen from these simulations that the observed affinity of agonists and inverse agonists in different expression systems can be likened to traveling on a curvilinear surface and that the magnitude of the affinity is dependent on systems effects such as receptor level, availability of G-protein and/or the level of spontaneous receptor activation (i.e., ionic effects vide infra).

Neutral antagonists are special entities. For a drug to qualify as such means that it must in no way modify the relative proportions of active and inactive receptor (no effect on receptor activation). This condition ($\alpha = 1$) requires that the ligand recognize no difference between the active and inactive conformations of the receptor, i.e., that it see both conformations identically. Secondly, the presence of the ligand must in no way alter the interaction of the receptor with G-protein (i.e., $\gamma = \delta = 1$); therefore, the ligand-bound receptor must adopt a conformation formally identical to the unbound receptor with respect to the binding of the G- protein. The ligand must also block the effects of an agonist. Only under these circumstances would the ligand qualify as a true neutral antagonist and would its affinity not be subject to systems effects (i.e., host cell type, receptor/G-protein stoichiometry, relative levels of receptor activation).

Neutral antagonists are of great value in receptor classification because they can be relied upon to chemically classify receptors in any host cell. It might also be supposed that true neutral antagonists might be less prevalent than previously thought and that the only reason that they appear to be so common in the literature is the fact that the existing test systems severely bias the observation toward neutral antagonism and not low levels of positive or negative efficacy. With the advent of constitutively active receptor systems (vide infra), many ligands thought to be neutral antagonists can be seen, in fact, to be inverse agonists. At this point, it should be stressed that the constitutively active receptor systems discussed here refer to those that demonstrate a truly spontaneously activated receptor and are not simply a system with an elevated baseline response. While all constitutively active receptor systems show elevated basal responses, there are other ways in which basal response can be elevated (i.e., release of endogenous agonist, vide infra).

In view of the strict thermodynamic requirements for neutral antagonism ($\alpha = \gamma = \delta = 1$), positive and negative efficacy may be thought to be a knife edge with ligands either stabilizing or destabilizing receptor/G-protein complexes. To what extent these nuances in coupling are pharmacologically relevant is as yet unknown. It is useful to differentiate constitutive activity and inverse agonism as a physiologically relevant phenomenon from its utility as a pharmacological looking glass into the properties of drugs. It is not at all clear that constitutive activity is prevalent in natural systems and that, therefore, inverse agonists will be therapeutically special. However, the availability of constitutively active receptor systems has allowed the reclassification of antagonists and has given new insights into 7TM receptor mechanisms. From this standpoint, this area of pharmacological research has proven to be useful.

The expectation of zero efficacy introduces the concept of the 'antagonist assumption' in receptor pharmacology (Kenakin et al., 1995). If a ligand is prematurely classified as a neutral antagonist on the basis of experiments in quiescent nonconstitutively active systems, then it automatically assumes an identity equal to that of other neutral antagonists. When such ligands are used to classify receptors and expression systems, then the tacit assumption is made that the ligands are all equal. Judgments as to the similarity of expression systems to natural systems of physiological interest are made on the basis of correlations of affinity of antagonists in the genetically created versus the natural system. If the ligands used for such correlations are *not* uniform (i.e., some are in fact inverse agonists and thus possibly subject to systems effects), then erroneous conclusions can be made. In these cases, it may be more warranted to reclassify the ligand rather than the receptor.

The previous discussions have defined a theoretical class of ligand, namely the inverse agonist. This entity destabilizes receptor/G-protein complexes, a property that is obvious only when receptor/G-protein complexes are present in such quantities as to be observed.

B. Detection of Inverse Agonism

Before the advent of drug testing in constitutively active receptor systems, drugs that blocked the effects of agonists but produced no positive response were classified as neutral antagonists. As in the case of ion channel two-state theory, if a system does not have spontaneous activity, then the effects of a drug that suppresses spontaneous activity will not be evident. Over the past few years, various methods of detecting such negative effects of drugs have been reported. In general, the main tenet of these approaches is that conditions are met for the increased prevalence of the spontaneously active receptor state, and/or the effects of the spontaneously active receptor state are amplified to the point at which they are observed.

One potential method of detecting inverse agonism is by increasing the basal activation of receptors. The first instance of biochemical detection of an inverse agonist for a 7TM receptor was reported by Costa and Herz (1989). They showed that the inverse agonist for opioid receptors ICI 174864 produced little negative effect on GTPase activity in NG108-15 cells until the constitutive GTPase activity of these membranes was elevated by substitution of NaCl by KCl (see fig. 6A). Spontaneous precoupling of opioid receptors and G-proteins is

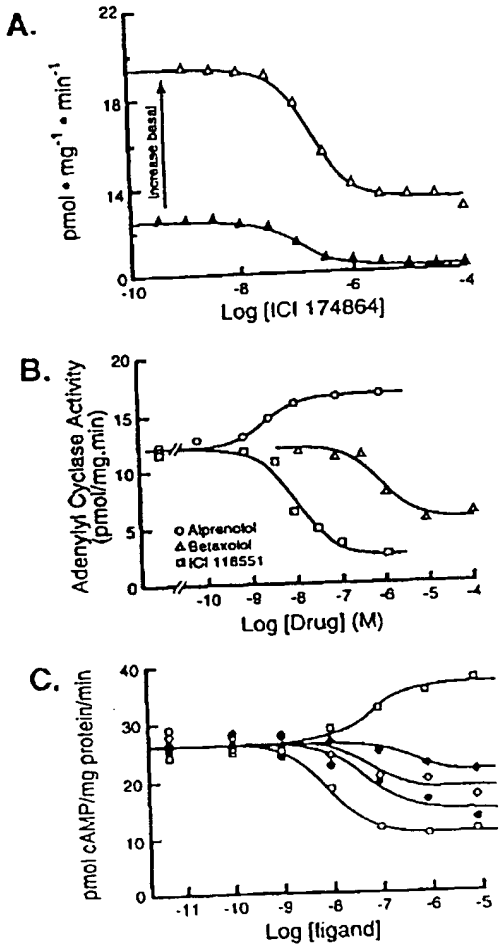


FIG. 6. Detection of inverse agonism. (A) Increased basal GTPase activity in membranes from NG108-15 cells by substitution of Na^+ with K^+ and the effects of the opioid inverse agonist ICI 174864 ([N,N'-diallyl-Tyr^1, Aib^2, 3]Leu^5-enkephalin). With permission from the National Academy of Sciences and from Costa and Herz (1989). (B) Effect of β-adrenergic receptor ligands on basal adenylate cyclase activity mediated by a constitutively-active mutant of the β_2-adrenergic receptor. With permission from ASPET and from Samama et al. (1994). (C) Effects of β-adrenergic blockers on basal adenylate cyclase activity in membranes from sf9 cells overexpressed with wild type β_2-adrenergic receptor. Responses to isoproterenol (open squares), dichloroisoproterenol (filled diamonds), labetolol (open diamonds), pindolol (filled circles), and timolol (open circles). With permission from ASPET and from Chidiac et al. (1994).

destablized by Na^+ (Costa et al., 1992). In addition, it is known that Na^+ modulates receptor affinity for G-proteins (Jagadeesh et al., 1990; Costa et al., 1990), and it is known that Na^+ produces dextral displacement of concentration-response curves to agonists for α_2-adrenergic receptors (Limbird et al., 1982), dopamine D_2 receptors (Hamblin and Creese, 1982) and δ-opioid receptors (Pert et al., 1973).

The original method reported by Costa and Herz (1989) has been extended to other receptors. Thus, removal of Na^+, shown to stimulate spontaneous association of G-proteins and opioid receptors (Costa et al., 1990, 1992), also produces constitutive activity for α_2-adrenergic receptors (Tian et al., 1994). Similarly, the binding of the α_2-adrenergic receptor inverse agonist [^{3}H]rauwolscine is increased 75% in PC-12 membranes with added Na^+ (Shi and Deth, 1994). There is evidence to suggest that, like GTP-induced cancellation of receptor/G-protein complexation, biochemical factors such as Na^+ may be important in the modulation of constitutive activity; although inverse agonism can be detected for some drugs in membrane systems in which the ionic milieu can be controlled, the same is not true in whole cell systems. Thus, the inverse agonism detected for some δ-opioid antagonists in membrane systems (Costa and Herz, 1989) was not observed in whole cellular systems (Costa et al., 1990). It may be that biochemical systems can be optimized for the detection of inverse agonism more easily than can functional systems.

Inverse agonists also have been discovered in binding studies by observing effects of guanine nucleotides. Thus, unlike the effects of GTP analogues on positive agonist binding (affinity is reduced), the binding of inverse agonists is *increased* by GTP. For example, studies on the reverse effects of GTPγS on binding have been used to detect negative efficacy in spiroperidol (De Lean et al., 1982). The adenosine receptor antagonist [^{3}H]xanthine amine congener preferentially binds to free adenosine receptors in bovine cerebral cortex (Freissmuth et al., 1991; Schutz and Freissmuth, 1992), where the receptors are spontaneously coupled to G-proteins (Leung and Green, 1989). This approach, although useful in some receptor systems, requires a kinetically favorable system for rapid exchange of guanosine diphosphate (GDP) to GTP. There are known G-protein systems that do not temporally respond adequately for this reaction to take place on an appropriate time scale, making this approach unreliable. This is discussed later in relation to agonist receptor coupling. A variant approach is by the cancellation of G-protein effects with toxins. Thus, pertussis toxin has been shown to *increase* the affinity of the opioid receptor inverse agonist ICI 174864 (Costa and Herz, 1989) and the α_2-adrenergic receptor inverse agonist rauwolscine (Jagadeesh et al., 1990; Shi and Deth, 1994).

Another approach is to study ligand effects on constitutively active mutant receptors. This has been used for the study of inverse agonism of β_2-blockers ICI 118,551 and betaxolol in CHO cells transfected with mutant constitutively active β-adrenergic receptors (See fig. 6B; Samama et al., 1994). Constitutively active mutants have been made also of α_{1B}-adrenergic receptors (Milano et al., 1994a) and α_2-adrenergic receptors (Ren et al., 1993). In general, the technical demands could limit the applicability of this technique. Also, the potential for differences between ligand activity on mutant versus wild type receptors always is open to question.

To date, the most fruitful approach is the testing of ligands in overexpressed receptor systems. There is evidence to suggest that the relative stoichiometry of receptors and G-proteins can be important in the production of constitutive activity. The concept here is that increasing the concentration of the reactants for spontaneous receptor/G-protein coupling will increase the quantity of activated receptor and G-protein and that this, in turn, will be observed either directly as GTPase activation or by subsequent activation of response elements (i.e., adenylate cyclase, etc.). Constitutive cellular activity has been shown to be related directly to β-adrenergic receptor expression levels in CHO cells (Samama et al., 1993), NG108-15 cells (Kim et al., 1995) and membranes from transfected sf9 cells (Chidiac et al., 1994). In CHO cells transfected for β-adrenergic receptor expression, the receptor density can be correlated with cellular basal adenylate cyclase activity. Thus, in clones expressing 2500 fmol/mg protein receptor, the basal level of cAMP approaches the maximally isoproterenol-stimulated levels in cells expressing 170 fmol/mg protein receptor (George et al., 1988). Figure 6C shows the positive and inverse effects of β-adrenergic receptor ligands on basal adenylate cyclase activity from membranes of sf9 cells transfected with high levels of β_2-adrenergic receptor. The stoichiometry also can be altered by increasing G-protein levels. For example, the reconstitution of dopamine D_2 receptors and G_{i2} proteins leads to spontaneous GTPase activity in the absence of agonist (Senogles et al., 1990).

Yet another approach is the testing of drugs in tissues from transgenic animals that produce constitutive activity by receptor overexpression. Transgenic mice (TG-4, TG-33) have been shown to demonstrate cardiac-specific overexpression of the wild type β_2-adrenergic receptor (Milano et al., 1994b). This receptor overexpression resulted in a three-fold increase in the baseline twitch tension of left atria that was selectively decreased by the β_2-adrenergic receptor inverse agonist ICI-118,551 (fig. 7A); this inverse agonism was blocked by the β_2-adrenergic receptor antagonist alprenolol (Bond et al., 1995). In vivo, this inverse agonism could be shown as well as decreases in left ventricular developed pressure (dp/dt); this effect also was blocked by alprenolol (fig. 7B). Identical responses could be seen with adenylate cyclase activity in membranes from TG-4 mouse hearts (fig. 7B).

There are practical and theoretical caveats to be made to the experimental support of inverse agonism. For example, the possibility of residual agonist present in bathing media of experimental preparations may demonstrate apparent inverse agonism for a neutral antagonist under partial agonist activation (i.e., Maenhaut et al., 1990). Also, the restriction of movement of agonist among free receptors in membrane systems may produce reduced G-protein activation (Mahama and Linderman, 1994). On balance, however, there are specific criteria for the demonstration of the data with

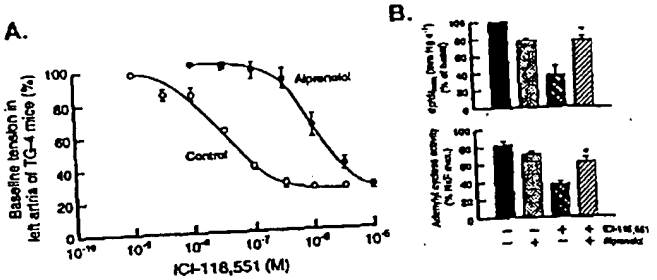


FIG. 7. Effects of inverse agonists in tissues from transgenic TG-4 mice with overexpressed cardiac β_2-adrenergic receptors. (A) Effect of the inverse agonist ICI-118,551 on baseline stimulated left atrial tension from TG-4 mice. Data from atria obtained in the absence (open circles) and presence of alprenolol (0.1 μM; filled circles). (B) Effects of ICI-118,551 on left ventricular dp/dt_{max} (upper bar graph) and adenylate cyclase activity (lower bar graph) in membranes from TG-4 mouse hearts. Although a minimal effect is seen with the neutral antagonist alprenolol, significant depression of constitutive activity is observed with ICI-118,551. This inverse agonism is reversed by alprenolol (1 μm). With permission from MacMillan Magazines and from Bond et al. (1995). (B) Effect of ICI 118,551 (5 μg i.v.) or alprenolol (10 μg i.v.) to mice ($n = 7$) (upper bars) and effect of 100 nM ICI 118,551 and alprenolol (1 μM) on adenylate cyclase activity. From Bond et al. (1995).

constitutively active receptor systems that have been met in many studies. They are as follows:

- Inverse effects have been observed in receptor systems with added G-protein (Schutz and Freissmuth, 1992).
- Reverse effects of GTP analogs and G-protein toxins have been noted on binding curves.
- Inverse agonism has been observed in transgenic animals.
- Inverse agonist responses can be blocked selectively by neutral antagonists (i.e., see fig. 7A).

This latter point is perhaps the most important. When this can be shown, it indicates that the phenomenon is a receptor mechanism.

With the development of sensitive test systems for the detection of inverse agonism will come a reclassification of many drugs. If it is accepted that efficacy is based on the differential affinity of a ligand for receptor states, then an expectation of 'zero' efficacy sometimes may be unrealistic because this would require identical affinities for two different tertiary forms of the receptor protein. Given this, it might be observed that numerous previously classified neutral antagonists may be inverse agonists. There are suggestions that this is true for β-blockers. For example four β-blockers tested in sf9 cells expressing β_2-adrenergic receptors produced inverse agonist effects (Chidiac et al., 1994) (fig. 6C). Similarly, Samama et al. (1994) found negative efficacy in two of three β-blockers tested (fig. 6B). In PC-12 cells expressing α_2-adrenergic receptors, five commonly used α_2-antagonists depressed [^{35}S]GTPγS binding indicative of inverse agonism (Tian et al., 1994). Table 9 shows a

TABLE 9

Putative inverse agonists: wild type receptors

Receptor	System	Drug	
β_2-Adrenergic	Sf9 membranes	DCI[a]	Chidiac et al., 1994
		pindolol	
		labetolol	
		timolol	
	CHW membranes	labetolol	
		pindolol	
		alprenolol	
		propranolol	
		timolol	
	turkey erythrocytes	propranolol	Gotze and Jakobs, 1994
		pindolol	
	TG-4 murine atria	ICI 118,551	Bond et al., 1995
β_1-Adrenergic	cardiomyocytes	atenolol	Mewes et al., 1993
		propranolol	
α_2-Adrenergic	PC-12 cells	rauwolscine	Tian et al., 1994
		yohimbine	
		WB 4101	
		idazoxan	
		phentolamine	
		yohimbine	
	bovine aorta	rauwolscine	Jagadeesh et al., 1990
	PC-12 cells	rauwolscine[b]	Shi and Deth, 1994
			Jagadeesh and Deth, 1992
Muscarinic Acetylcholine	frog/rat atrial myocytes	atropine[c]	Hanf et al., 1993
	frog heart	QNB[d]	Burgisser et al., 1982
Bradykinin	Rat myometria	HOE140	Leeb-Lundberg et al., 1994
		NPC17731	
		NPC567	
5-HT_{2C}	NIH 3T3 cells	mianserin	Barker et al., 1994
		mesulergine	
		ketanserin	
	NIH 3T3 cells	clozapine	Westphal and Sanders-Bush, 1994
		cyproheptadine	
		ketanserin	
		mesulergine	
	Sf9 cells	metergoline	Lebrecque et al., 1995
		methysergide	
		ritanserin	
		mianserin	
		mesulergine	
		ketanserin	
		clozapine	
		spiperone	
δ-Opioid	NG108-15 cells	ICI 174864	Costa and Herz, 1989
Dopamine[e]	P. anterior pituitary	spiroperidol	De Lean et al., 1982
Adenosine[e]	Bovine brain	XAC	Freissmuth et al., 1991
	Constitutively active mutants		
β_2-Adrenergic	CHO membranes	betaxolol	Samama et al., 1994
		ICI 118,551	
α_{1B}-Adrenergic	Rat-1 Fibrob.	prazosin	Cotecchia et al., 1995
		WB 4101	
		phentolamine	

[a] Dichloroisoproterenol.
[b] Increased binding in the presence of added Na^+.
[c] At 1 μM.
[d] [^{3}H]Quinuclidinyl benzoate.
[e] Suggested by the increased binding observed with inclusion of GTP in medium.

list of possible inverse agonists, previously thought to be neutral antagonists from data in quiescent systems. It should be noted that in many of the cases cited, blockade of the inverse effect with a neutral antagonist was not shown; therefore, the data are consistent with but not necessarily proof of true inverse agonism.

Presently, it is unclear to what extent, if any, inverse agonists will affect therapeutic approaches to 7TM re-

ceptors. Clearly, an inverse agonist will have a somewhat different profile of antagonism across various tissues in the body than a neutral antagonist in that they will block endogenous agonist *and* any constitutive receptor activity, whereas the latter will only block the effects of endogenous agonists. Insofar as receptor overexpression may lead to constitutive basal activity in tissues, an inverse agonist would be a unique drug. For example, dopamine D_4 receptors are elevated six-fold over control in patients with schizophrenia (Lee et al., 1978; Seeman et al., 1993). If this increase in receptor density leads to constitutively active foci of activity, these would be resistant to standard dopamine antagonists such as haloperidol but sensitive to negative antagonists. Also, there is evidence that 7TM receptor mutation sometimes may lead to a pathologically relevant, constitutively active receptor mutants (vide infra). Under these circumstances, an inverse agonist would be needed. At present, it is premature to speculate on the potential merits of inverse agonists (Milligan et al., 1995a); however, in view of the fact that they are a newly discovered drug type, it will be extremely interesting to see what place they find in therapy.

It may be advantageous to detect inverse agonism in antagonists for other reasons. For example, a theoretical case could be made for a greater risk of receptor up-regulation (and therefore, of tolerance to blockade) for inverse agonists versus neutral antagonists (Milligan et al., 1995b). If normal levels of receptor on the membrane are controlled by phosphorylation of spontaneously activated receptors, then an inverse agonist could prevent this normal process and thus produce an imbalance in the receptor synthesis/destruction cycle.

C. Receptor Expression Levels and Relative Stoichiometry

1. Agonist coupling. Heterologous expression of 7TM receptors particularly tests the assumption that high agonist affinity binding will be observed in surrogate cell systems. In some expression systems, there is reasonable correspondence between the type and quantity of high affinity binding observed in natural systems and heterologous expression systems. For example, ^{125}I-VIP demonstrates high (K_d = 0.3 nM) and low (K_d = 23 nm) binding in rat lung, with a relative proportion of 13% high affinity sites (Leroux et al., 1984). In COS cells transfected for expression of rat VIP receptors, similar complex ^{125}I-VIP binding could be observed (high affinity K_d = 0.16 nM, low affinity K_d = 20 nM, 7% high affinity sites: Ishihara et al., 1992). One novel approach has been the study of radioligand binding to the effector to detect ternary complex formation. For example, increased binding of [^{3}H]forskolin to adenylate cyclase in response to $G_{\alpha s}$ and transfected receptor (prostanoid, β-adrenergic) interaction in NG108-15 cells provided insight into the stoichiometry of receptor-effector relationships (Kim et al., 1995).

The definition of a 'high-affinity' site for a ligand often is subjective when agonist radioligands are used. Commonly, a single population of binding sites is observed because accurate binding data at the high concentrations of radioligand required to define low affinity binding (i.e., the uncoupled form of the receptor) is unobtainable. Thus, if a single homogeneous population of receptors is observed with an agonist radioligand, it is not possible to determine whether this is a completely coupled ternary complex binding product or an uncoupled receptor unless an antagonist radioligand is used to determine the receptor population size. For example, saturation binding of transfected rat 5-HT_7 receptors in COS-7 cells yields a population size of 5 to 15 pmol/mg protein when measured with the antagonist [^{3}H]lysergic acid diethylamide and 2 to 10 pmol/mg protein when measured with the agonist [^{3}H]5-HT (Shen et al., 1993). These data suggest the labeling of a coupled receptor subset with the agonist.

The determination of G-protein binding by elimination of high affinity sites with analogues of GTP (i.e., Gpp(NH)p) is one potential method of determining whether observed high affinity binding is because of a two-stage process of receptor/G-protein coupling. Some of these effects can be striking, as in the 1480-fold change in affinity of carbachol in rat heart membranes produced by GTP (Matesic et al., 1989). Such studies have also been done in membranes from surrogate cells transfected with receptor genes. For example, the high affinity binding of [^{3}H]5-HT to human 5-HT_{1B} receptors in HeLa cells (Hamblin et al., 1992) and rat 5-HT_1 receptors in HEK 293 cells (Voigt et al., 1991b) is greatly reduced by inclusion of analogues of GTP. Similar sensitivity of complex agonist binding to GTP analogues has been observed for rat α_2-adrenergic receptor subtypes α_{2B} and α_{2D} (but not α_{2C}) in NIH 3T3 fibroblasts (Duzic et al., 1992), rat dopamine D_2 and D_3 receptors expressed in various cell lines (Sokoloff et al., 1990; Castro and Strange, 1993), rat adenosine A_1 receptors in A-9 cells (Mahan et al., 1991), human adenosine A_1 receptors in CHO cells (Libert et al., 1992), human 5-HT_{1D} receptors (Hamblin and Metcalf, 1991), human bradykinin B_2 receptors (Hess et al., 1992), human somatostatin receptors (Demchyshyn et al., 1993), mouse β_2-adrenergic receptors (Allen et al., 1988), human 5-HT S12 receptor (Levy et al., 1992), human dopamine D_4 receptor (Van Tol et al., 1991), human cholecystokinin (CCK)-B receptor (Miyake et al., 1994) and the guinea pig leukotriene B_4 receptor (Falcone and Aharony, 1991).

On the other hand, the cancellation of receptor coupling by GTP is a one-way experiment in that, if high affinity binding is eliminated, G-protein binding is implied, but if high affinity binding is not eliminated, other factors, such as kinetics of GDP/GTP exchange, may have confounded the experiment. Under these circumstances, it cannot be assumed that G-protein coupling is not present. For example, in Y-1 cells transfected with

genes encoding the mouse m1 muscarinic acetylcholine receptor, carbachol produces a clearly seen population of high affinity binding sites (26% pK_H = 5.4, 74% pK_L = 3.9) that are resistant to treatment of the membranes with Gpp(NH)p (Shapiro et al., 1988). The same results were obtained for the chick muscarinic acetylcholine receptor expressed in CHO cells, namely a lack of effect of Gpp(NH)p on complex inhibition of [^{3}H]QNB binding by carbachol (Tietje et al., 1990). Similarly, the binding of the agonist [^{3}H]5-HT to rat 5-HT_7 receptors transfected into COS-7 cells is unaffected by GTP (Shen et al., 1993). An interesting contrast was observed in CHO cells transfected with genes for human D_2 and D_3 receptors, respectively. Although high affinity dopamine binding was sensitive to Gpp(NH)p in D_2 receptor transfected cells, high affinity binding of dopamine to D_3 receptors in the same cell line was resistant to this procedure (Sokoloff et al., 1992). Similar heterogeneity of GTP-effects was observed for 5-HT_2 receptors transfected into HEK 293 and NIH 3T3 cells. Whereas agonist coupling was insensitive to GTP analogues in HEK 293 cells, it was partially sensitive in NIH 3T3 cells (Szele and Pritchett, 1993).

Variability in the 'GTP-shift' has been analyzed in a model proposed by Onaran and coworkers (1993), who extended the ternary complex model to include the dissociable subunits of the G-protein and the effects of nucleotides. In general, this model predicts large differences in the observed effects of nucleotides on ligand binding that depend on system characteristics, namely the coupling factor modifications of unconditional equilibrium constants among the G-protein subunits, the receptor and the α-subunit. As shown in figure 8A and B, the concentration of $\beta\gamma$ subunits affect the observed potency of agonists differentially under conditions of high to low GTP. The difference between these curves (at a fixed quantity of $\beta\gamma$ subunit in the system) is the 'GTP shift,' and it can be seen that the magnitude of this shift is determined by the availability of $\beta\gamma$ subunit. Interestingly, this model also predicts that the availability of $\beta\gamma$ subunits also affects spontaneous constitutive and agonist-induced receptor activation. As seen in figure 8C, constitutive activity is more sensitive to the concentration of $\beta\gamma$ subunits than is agonist activation. This has been observed experimentally as well (Cerione et al., 1985; Okabe et al., 1990; Hildebrandt and Kohnken, 1990). As seen in figure 8D, where the basal effect is subtracted, the concentration of $\beta\gamma$ subunits has a biphasic effect on agonist activation, increasing it at lower concentrations and then decreasing it at higher concentrations. Thus, the $\beta\gamma$ subunits buffer the system for both spontaneous activity and agonist effect. The importance of $\beta\gamma$ subunits (a cellular host effect) has been shown in antisense oligonucleotide experiments where certain β-subunits were found to be involved in signal transduction cascades in GH3 cells (Kleuss et al., 1992). Recent provocative data with $\alpha_{2A/D}$-adrenergic receptors

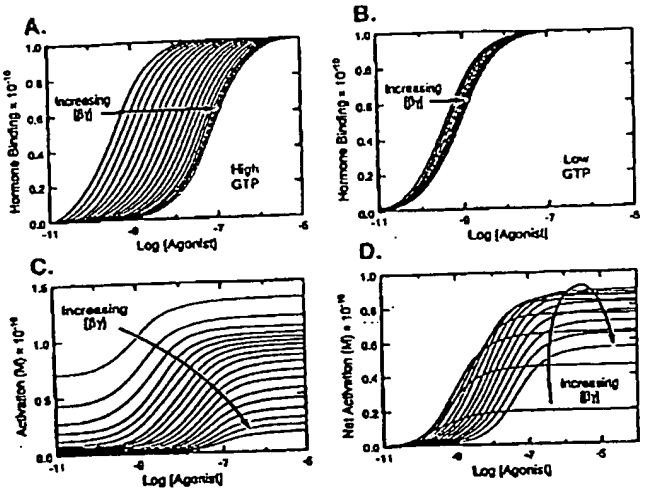


FIG. 8. The effects of $\beta\gamma$ subunits on high affinity ligand binding and receptor activation. (A) Simulation of high affinity binding with various concentrations of $\beta\gamma$ subunits in the presence of high concentrations of GTP and in the presence of low GTP (panel B). The relative locations of the dose-response curves for any given concentration of GTP reflects the GTP-shift induced by GTP, indicating agonist efficacy and receptor/G-protein coupling. (C) Effects of $\beta\gamma$ subunits on receptor activation. Note how increasing $\beta\gamma$ concentration depresses constitutive activity, changes maximal responses to an agonist and decreases sensitivity of the system to the agonist, but that each of these systems characteristics is not equally sensitive to $\beta\gamma$ concentration. (D) Simulation shown in C with the agonist-independent (constitutive) activity subtracted. This shows the biphasic effect of $\beta\gamma$ concentration on the maximal effect of an agonist with an intial increase then decrease with increasing $\beta\gamma$ concentration. Data from Onaran (1993).

in NIH 3T3 and PC-12 cells provide evidence for the involvement of a specific membrane-associated protein in the interaction of agonist-induced activation of G-proteins (Sato et al., 1995). A similar protein has been found for adenosine receptors. This factor causes tight receptor/G-protein coupling that is refractory to GTPγS (Nanoff et al., 1995). The implications of another membrane interactant in the receptor cascade are extremely important in terms of defining and quantifying agonist efficacy for drug therapy.

The expectation of observing a high affinity agonist binding in an expression system presupposes that the appropriate G-protein is present in the expression cell and also that it is there in sufficient quantities to produce observable ternary complexation. This can vary with different expression systems as was illustrated in studies of the expressed rat 5-HT_{5B} receptor. Accordingly, a high and low affinity state for this receptor for a 5-HT agonist that was greatly reduced by the presence of the nonhydrolyzable GTP analogue Gpp(NH)p (showing receptor/G-protein coupling) could be demonstrated when expressed in COS-1 cells; no corresponding G-protein activation could be demonstrated when this receptor was transiently expressed in COS-7, COS-293 or CHO cells (Wisden et al., 1993). Clearly, if the appropri-

ate G-protein is not available in the receptor compartment, then the uncoupled (and presumably low affinity) state of the receptor will be present, and technical considerations may preclude the observation of agonist binding with radioligands. In cellular expression systems in which the stoichiometry of receptor and G-proteins becomes a variable, the 'overexpression' of receptors can cause conditions whereby the ratio of uncoupled to coupled receptor is large, giving the appearance of a failure to G-protein couple the expressed receptors. For example, rat 5-HT_2 receptors expressed in mammalian cells show high affinity binding for the antagonist [^{3}H]spiperone but no detectable binding of [^{3}H]5-HT (Pritchett et al., 1988). A list of receptors demonstrating a range in proportions of receptor coupling efficiencies in expression systems is shown in table 10. It can be seen from this list that as long as an adequately strong signal can be obtained from the bound radioligand, exceedingly small percentages of coupled receptor can be detected.

The observation of G-protein coupled receptor events also may differ in binding and functional systems. There are cases where the biochemical amplification of minute G-protein signals allow agonist activation to be observed (i.e., cellular response), but where the amount of G-protein isomerization of the receptor is insufficient to be observed with ligand binding. For example, the adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA) produces a clear increased cAMP response in CHO-K1 cells transfected with a cDNA for adenosine receptors from human brain, but no [^{3}H]NECA binding was observed (Pierce et al., 1992). Presumably, a low level of G-protein activation was sufficiently amplified by the biochemical cascade mechanisms in the cell to produce a measurable response, but there was an insufficient quantity of receptor distribution into a ternary complex to allow for the observance of high affinity agonist binding.

2. *Relative expression level and promiscuity of coupling.* In natural systems, the stoichiometry of receptors and G-proteins is fixed by nature. With receptor expression comes two new potential phenomena, namely the induction of constitutive receptor activation and also increased receptor promiscuity with respect to activation of numerous G-proteins. Increased complex receptor coupling with increased levels of receptor expression is becoming a commonly observed experimental phenomenon.

TABLE 10

Two affinity states for agonists in expression systems

Receptor	Cell	Agonist	pK_H	pK_L	%H/%L	Reference
Agonist Saturation Binding in Expression Systems						
r 5-HT_1	HEK 293	[^{3}H]5-HT	8.4	7.2	NG	Voigt et al., 1991b
h 5-$HT1_B$	sf9	[^{3}H]5-HT	11	10	50/50	Ng et al., 1993
r 5-HT_{1A}	Ltk$^-$	[^{3}H]DPAT	8.8	8.0	50/50	Albert et al., 1990
r α_{2D}	NIH 3T3	[^{3}H]UK 14,304	8.15	5.85	37.5/62.5	Duzic et al., 1992
m 5-HT_{1B}	sf9	[^{3}H]5-HT	11	9.9	36/64	Ng et al., 1993
r α_{2B}	NIH 3T3	[^{3}H]UK 14,304	8.3	6.0	34/66	Duzic et al., 1992
h 5-HT_{1B}	HeLa	[^{3}H]5-HT	9.27	8.1	30/70	Hamblin et al., 1992
r secretin	COS + G_s[a]	^{125}I-secretin	9.2	7.7	15/85	Ishihara et al., 1991
h 5-HT_{1D}	LS12/6.2	[^{3}H]5-HT	7.7	6.7	11.8/88.2	Levy et al., 1992
r VIP	COS	^{125}I-VIP	9.8	7.7	7/93	Ishihara et al., 1992
h 5-HT_A	COS-7	[^{3}H]DPAT	10.2	7.65	2.6/97.5	Fargin et al., 1988
r secretin	COS	^{125}I-Secretin	9.2	7.7	1.8/98.2	Ishihara et al., 1991
Agonist Inhibition Binding of Antagonist Radiolabels						
m musc m1	CHO	carbachol	5.26	3.9	85/15	Shapiro et al., 1988
c musc m4	CHO	carbachol	5.27	3.9	85/15	Tietje et al., 1990
r A1	A-9	r-PIA	9.15	7	72/28	Mahan et al., 1991
m musc m1	Y-1	carbachol	5.5	4.2	70/30	Shapiro et al., 1988
r D_{2short}	LZRI	dopamine	8.1	6.1	58/42	Castro and Strange, 1993
r D_{2long}	CHO	dopamine	7.5	5.45	48/52	Castro and Strange, 1993
h D_3	CHO	dopamine	8.4	7.1	47/53	Sokoloff et al., 1992
r D_3	CHO	dopamine	8.6	7.2	40/60	Castro and Strange, 1993
h D_2	C6 cells	dopamine	7.66	5.8	34/66	Sokoloff et al., 1992
rh D_1	HEK	carbachol	8	6	32/68	Machida et al., 1990
h m1	HEK	carbachol	4.1	3.3	30/70	Peralta et al., 1987
h m2	Ltk59	dopamine	7	3.3	28/72	Peralta et al., 1987
r D_{2long}	HEK	oxotremorine	8.1	6	25/75	Castro and Strange, 1993
h m2	HEK	carbachol	8.1	5.3	20/80	Peralta et al., 1987
h m4	HEK	oxotremorine	5.1	3.25	6.5/93.5	Peralta et al., 1987
h m3			7.66	5.4	5/95	Peralta et al., 1987

[a] Cotransfected with G_s.

r, rat; h, human; m, mouse; c, chick; rh; rhesus monkey; VIP, vasoactive intestinal peptide; r-PIA, r-phenylisopropyladenosine.

There is an intrinsic association constant between receptors and all G-proteins (i.e., K_G in the cubic ternary complex model), and selectivity of receptor/G-protein coupling can be achieved by this K_G and the relative 'concentrations' of the receptor and G-protein (i.e., very little receptor/G-protein complex will be formed by a receptor with an association constant K_G of 10^4 at a concentration of 10 μM). However, if the concentration of receptor were increased 100-fold, then an appreciable amount of even this unfavored complex will be formed.

If it is accepted that receptors can be promiscuous with respect to the G-proteins that they encounter in the membrane, then there is a potential for dissimulation of effect with receptor overexpression. One method of achieving signaling selectivity in nature is to control the stoichiometry of receptors and G-proteins; if this is overridden in a heterologously expressed system, then system-dependent data may result that may not reflect the physiology of the receptor. For example, receptor expression level has been shown to determine the cellular responses mediated by transfected α_2-adrenergic receptors in CHO cells (Fraser et al., 1989). Thus, in cells containing 50 fmol/mg protein, primarily inhibition of cAMP levels was observed with epinephrine, whereas in cells containing 1200 fmol/mg protein, a biphasic inhibition and stimulation of cAMP level was seen. The inhibition phase was sensitive to treatment of cells with pertussis toxin, suggesting that this receptor activated two separate G-proteins in CHO cells (Fraser et al., 1989). Similarly, muscarinic receptors expressed in JEG-3 cells can either inhibit or stimulate adenylate cyclase, the nature of the response being dependent upon the receptor expression level (Migeon and Nathanson, 1994). Another example of receptor density controlling the effect in a cellular system is with the expression of α_{2A}-adrenergic receptors in COS cells (Eason et al., 1992). In this study, an α_2-adrenergic receptor level of 1 pmol/mg protein yielded a system that mediated inhibition of adenylate cyclase (via G_i protein), whereas higher receptor expression levels (5 and 10 pmol/mg protein) produced biphasic interaction of the receptor with both G_i and G_s to inhibit and then subsequently stimulate adenylate cyclase. The α_{2A}-adrenergic receptor expressed in Rat-1 fibroblasts has been shown to activate several cellular elements including phospholipase D (MacNulty et al., 1992). Similarly, expression of muscarinic m4 receptors in HEK 293 demonstrated a biphasic response with respect to adenylate cyclase activity that depended upon receptor number. Thus, in cells with low levels of receptor expression, activation of m4 receptors resulted in inhibition of cAMP, whereas in cell lines exhibiting the highest levels of receptor expression, a biphasic response of inhibition and stimulation was observed. This was consistent with receptor competition with a pertussis-sensitive and -insensitive G-protein in the cell membrane (Dittman et al., 1994). In CHO cells transfected with muscarinic m3 receptors, carbachol produced both increased accumulation of inositol 1,4,5-triphosphate and cAMP (Burford et al., 1995). However, a 50% reduction in the receptor transfection level resulted in loss of cAMP response.

An interesting difference in signaling was shown for the human calcitonin receptor. Expression of the cDNA, obtained from T47D cells, in BHK cells led to a receptor profile similar to that found in T47D cells. However, the function of the expressed system differed. Although calcitonin increased intracellular calcium, inositol phosphate production and cAMP in BHK cells, only the cAMP response was observed in T47D cells (Moore et al., 1992). This difference in coupling may have been related to the difference in receptor densities in the two cell types (BHK cells 800,000 receptors/cell; T47D 40,000 receptors/cell).

Another case of receptor-coupling promiscuity tied to receptor expression level was observed in African green monkey cells (BS-C-1) transfected with human 5-HT_{1E} receptors. While low expression levels (2 pmol/mg protein) produced 5-HT-mediated inhibition of cAMP production, higher levels (5 pmol/mg protein) produced cells showing a biphasic decrease and increased cAMP response to this agonist (Adham et al., 1994b). Each respective response could be eliminated by treatment of cells with pertussis and cholera toxins(s), indicating promiscuous coupling of this expressed receptor to separate G-proteins.

There are theoretical reasons for concern over receptor density versus availability and type of G-protein coupler present in the membrane. Figure 9 shows the results of modeling using the cubic ternary complex model with one receptor binding to two G-proteins (Kenakin and Morgan, 1989). The simulation is for the maximal ternary complex produced by an agonist that activates a single receptor toward interaction with two G-proteins. G-protein G_1 is favored and is the primary physiological coupler for the receptor-agonist pair, but there is a weak interaction between the agonist-activated receptor and the second G-protein G_2. As can be seen from figure 9, at receptor levels from 0.01 to 10, essentially all of the ternary complex is formed with G_1. However, as the quantity of receptor increases beyond this level, appreciable levels of ternary complex with G_2 can be observed. The resulting cellular response resulting from the biochemical cascades initiated by both G_1 and G_2 might be expected to be quite different when compared with the result emanating from only G_1; thus, a qualitative and quantitative difference in agonist response probably would be seen as a function of receptor expression level (Kenakin, 1995a).

D. The Nature of Efficacy: Receptor Activation

There has been much effort placed into the study of how receptors can activate G-proteins and effectors. As discussed previously, theoretical and practical data suggest that an active conformation of the receptor can bind

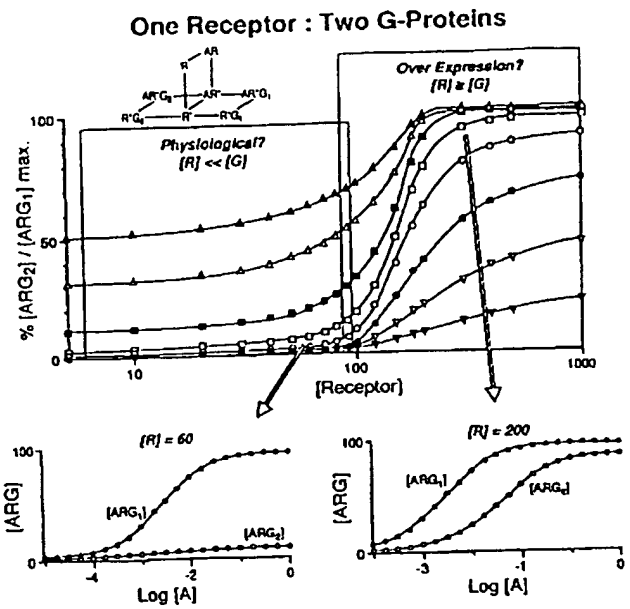


FIG. 9. Simulation of a single receptor plus two-G-protein membrane system. Ordinate axes show the maximum quantity of ternary complex formed between agonist/receptor and primary G-protein G_1 (right-hand axis) and agonist/receptor and secondary G-protein G_2 as a function of receptor density (abscissae). The relative concentration of G_1/G_2 is 10 to 20. Other system-dependent parameters are: [R] = variable, J = 0.1, β = 1000: Agonist dependent parameters = M_1 = 0.01, α_1 = 1000, M_2 = 0.01, α_2 = 10 (see schematic inset). The agonist promotes a preferential ternary complex with G_1. As can be seen from this figure, at receptor concentrations from 3 to 50, essentially all of the ternary complex is formed with G_1 (binding curve inset for [R] = 60). However, as the receptor concentration is increased beyond 50, a second complex of agonist/receptor/G_2 appears with increasing prevalence (binding curve inset for [R] = 200).

to G-proteins and begin the process of effector activation. There is evidence that short synthetic peptides from the third and fourth intracellular loops of G-protein receptors in close apposition to the membrane are known to stimulate G-proteins in vitro (Okamoto et al., 1991; Cheung et al., 1991; Ikezu et al., 1992). This would suggest that inactive receptors have these binding domains inaccessible to G-proteins and that agonists 'relax' the receptor to expose these domains and thus initiate activation (Lefkowitz et al., 1993). Under these conditions, inactive receptors could be thought to be under tonic constraint with respect to these intracellular domains (Lefkowitz et al., 1993). There is evidence that mutations of some receptors in specific regions produce constitutively active receptors and that the inactive receptor is the exception, not the rule. For example, substitution at position 293 of the α_{1B}-adrenergic receptor with any one of 19 other amino acids (different from the wild type) produces a receptor that spontaneously produces inositol phosphate production (Kjelsberg et al., 1992). In the bacterial chemoreceptor Trg, 20 mutations led to nine constitutively active receptors and 11 quiescent ones (Yaghmai and Hazelbaurer, 1992). Thus, in general terms, it may be that the inactive conformation of the receptor is the special one, designed to keep inaccessible the G-protein-activating amino acid sequences, and that deviation from such conformation(s) leads to a partially or completely activated receptor.

A central question in pharmacology is the nature of agonist efficacy, i.e., what makes agonists enrich the membrane population of activated receptors? A useful early delineation of ideas was proposed by Burgen (1966), who suggested that receptors could impart signals either by 'conformational selection' or 'conformational induction.' The first idea describes a condition whereby the receptor pre-exists in at least two states, one of which elicits cellular signaling (the activated receptor state). Agonists selectively bind to this activated state and enrich the population and thus produce a drug-induced response. The second idea describes an active receptor conformation created by the agonist and thus not present in the absence of the agonist.

Although it necessitates at least two receptor states, the most parsimonious hypothesis is conformational selection because it does not require additional receptor conformations other than those that exist naturally. There is evidence that naturally activated receptors exist and can activate a variety of effector systems. In fact, there is evidence to show that receptors can form different conformations and display complex binding kinetics in receptor systems stripped of G-proteins. Thus, muscarinic receptors, solubilized with digitonin-cholate and further processed to remove G-proteins displayed biphasic binding curves for the agonist oxotremorine (Wreggett and Wells, 1995). These data can be described with a tetravalent oligomeric receptor model that involves different receptor conformations not dependent upon G-proteins (Wreggett and Wells, 1995).

In terms of the concept of receptors existing in different conformations, selective binding to the activated form of the receptor by a ligand will enrich the relative proportion of that activated species and produce response (i.e., fig. 2). There are data becoming available to probe the nature of the receptor species responsible for physiological response. The most valuable systems to explore this area are those in which a single receptor species is capable of interacting with two or more G-proteins. The relative activation of the G-proteins involved may provide an insight into the activated receptor species formed by agonists.

1. Receptor trafficking of stimulus. There is evidence that some agonists specifically direct receptor signaling traffic toward specific G-proteins making agonist responses selective for receptor/G-protein combinations (shown schematically in fig. 10A). For example, in CHO cells transfected with 5-HT_{1A} receptors, a range of full and partial agonists differentially produced activation of $G_{\alpha i2}$ and $G_{\alpha i3}$. Whereas 5-HT appeared to be equiactive for G-proteins, the agonist ipsapirone showed a distinction (Gettys et al., 1994). Agonists for m1 acetylcholine

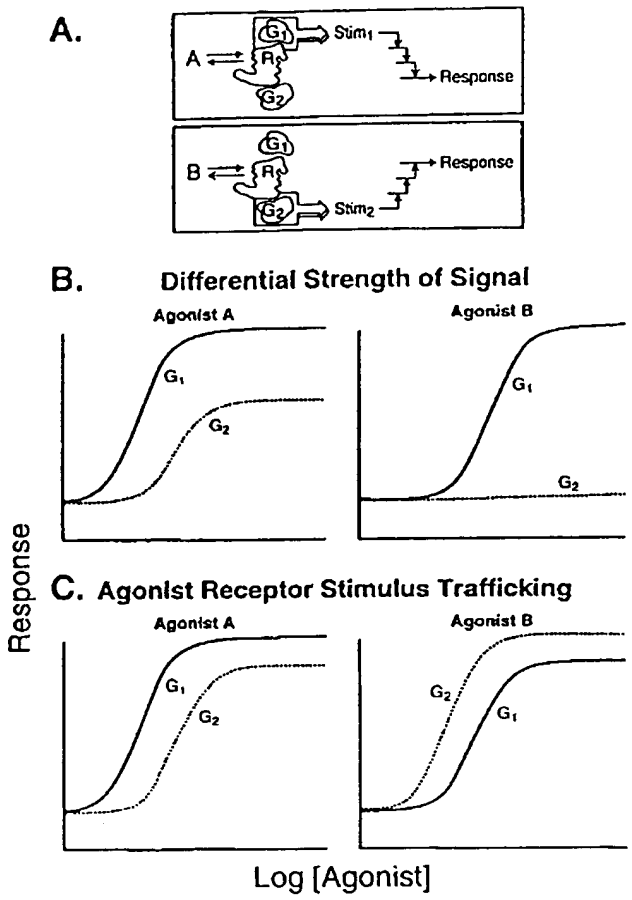


FIG. 10. Agonist trafficking of receptor stimulus to different G-proteins. (A) Schematic diagram of the concept of receptor stimulus trafficking by agonists. Agonist A produces response by directing receptor activation to G-protein 1 while agonist B uses another G-protein. Presumably, this is caused by differences in the conformation of the activated state either stabilized by or created by the different agonists. From Kenakin (1995b). (B) Simulated data consistent with differences in G-protein activation by differential strength of signal. Agonist A is highly efficacious and activates G_1 and the less sensitive G_2, whereas agonist B is weaker and can only activate the most sensitive process, namely G_1. (C) Data simulating true agonist directed trafficking of receptor stimulus. Agonist A preferentially activates G_1, whereas agonist B preferentially activates G_2.

(m1AChR) receptors also may direct trafficking to selective biochemical cellular pathways. In CHO cells transfected with m1AChR, quantitative differences in the potencies of carbachol, pilocarpine and AF102B for activation of phosphoinositide hydrolysis, arachidonic acid release and cAMP accumulation indicate selective activation associated with receptor recognition of ligands (Gurwitz et al., 1994). A clear distinction between G-protein activation of G_s and G_i by α_2-adrenergic receptors was shown by oxymetazoline and epinephrine in CHO cells (Eason et al., 1994). Whereas epinephrine activated both G-proteins in a similar concentration range, oxymetazoline could be shown only to activate G_i.

Another possible indication of agonist directed trafficking comes from the observation of high and low affinity binding states with different agonists in various expression systems. For example, carbachol and oxotremorine are known agonists for human muscarinic acetylcholine receptors. Transfection of subtypes 1 to 4 into HEK cells leads to interesting differences in high and low affinity inhibition of [^{3}H]QNB binding. Specifically, whereas the m2 subtype shows comparable high and low affinity states with both agonists, oxotremorine exclusively produces two states in cells transfected with subtype m3, and carbachol exclusively produces two states for transfections with subtype m1 and m4 (Peralta et al., 1987). Although the ability to produce a demonstrable high affinity state is dependent on the intrinsic efficacy of the agonist, these data suggest that whatever G-proteins are available in the HEK cell for complexation with the receptors are differentially used by the agonists in producing coupling states.

One possible explanation for these data is that the various agonists produce different activated receptor conformations that have different relative affinities for G-proteins, i.e., these agonists 'traffic' the receptor stimulus toward different G-proteins. At this point, the nature of the receptor species that activates the G-protein should be defined. At present, there is an abundance of evidence that different regions of 7TM receptors activate different G-proteins (i.e., the same sequences do not universally activate all G-proteins) and that selectivity for G-protein coupling can result (Wong et al., 1990; Kosugi et al., 1992; Okamoto and Nishimoto, 1992; Yamada et al., 1994; Nussenveig et al., 1994; Wu et al., 1995). However, it is not clear whether the activated receptor exposes all or just some of these upon conformational change to the active state. There is suggestive biochemical evidence to indicate that agonist-bound receptor complexes differ from those not containing agonist with respect to G-protein binding. For example, whereas some antisera for α-subunits of G-proteins do not differentiate spontaneously receptor-bound G-proteins and those produced by agonists, the amount of spontaneously coupled α_2-adrenergic receptor to $G_{\alpha i}$ is reduced by the α_2-adrenergic receptor agonist p-aminoclonidine (Okuma and Reisine, 1992). This indicates that the receptor complex spontaneously coupled to this G-protein and the activated receptor formed by p-aminoclonidine were seen to be different by the G-protein (as indicated by immunoprecipitation with the antiserum).

Although there is suggestive evidence, there is still a paucity of definitive data to indicate that agonist-selective activated receptor complexes exist. Also, before this complex hypothesis can be considered, the more simple scenario of selective G-protein activation graded by strength of stimulus must be eliminated. This idea states that the spectrum of G-protein activation is produced by the actual strength of stimulus, in the case of

receptor conformational selection, by the actual quantity of activated receptor state. Thus, a powerful agonist that produces a great deal of activated receptor will activate many G-proteins, whereas a weaker agonist will only produce enough activated receptor to activate the most efficiently coupled G-protein (i.e., highest K_G). For example, although carbachol, pilocarpine and McN A343 are all muscarinic receptor agonists, it can be shown that they produce a spectrum of maximal amounts of G-protein activation by immunoprecipitation (Matesic et al., 1991).

In general, cellular cascades consisting of sequential saturable biochemical reactions lead to progressive amplification of receptor stimuli. Under these conditions, it is possible that a given receptor stimulus will be of sufficient strength to trigger another signal in the cytosol. The relevance to receptor classification is the possibility that the strength of signal may determine the pleiotropy or lack of it, i.e., whereas a strong efficacious agonist may trigger many biochemical cascades in the cell, a weaker one (partial agonist) may induce only the most sensitive and highly amplified one. For example, the opioid agonist DADLE stimulates high affinity GTPase and also inhibits basal adenylate cyclase in NG108-115 cells. However, upon reduction of receptor stimulus through alkylation, the less sensitive response (GTPase) is eliminated, and the more sensitive one remains (Costa et al., 1988). Similar effects were observed by Saussy et al. (1989) who showed that the partial LTD4 receptor agonist LTE4 activated only a portion of the signaling system available to the receptor when it was activated by LTD4 in U-937 cells. In liver membranes, glucagon has been shown to activate adenylate cyclase as well as elevate IP_3, whereas the partial agonist des-His^1[Glu^9] glucagon amide only elevated IP_3 (Unson et al., 1989). In general, the simple demonstration of multiple versus single activation of biochemical pathways cannot be used as definitive evidence of differences of agonist effect at the receptor (see fig. 10B).

The possibility of selective G-protein activation by strength of stimulus always exists in systems where some agonists activate numerous G-proteins and others only a few. However, if the relative potency of selective G-protein activation could be shown to be different for different agonists, this would truly imply that the agonists concerned produced selective receptor activation states (see fig. 10C). For example, the PACAP receptor PACAP-R transfected into LLC PK1 cells mediates stimulation of cAMP levels and inositol phosphate production (Spengler et al., 1993). However, the relative potency of the agonists PACAP-38 and PACAP-27 is reversed for these two responses, indicating that some preferential ternary complex was formed for each agonist. Similar data have been reported for octopamine/tyramine receptors in *Drosophila*, where a clear reversal of potency for cAMP attenuation and Ca^{2+} transients is seen for octopamine and tyramine (Robb et al., 1994).

In addition to the reversal of agonist potency suggesting that stimulus trafficking can be produced by some agonists, study of the interactions of receptors with different G-proteins can be useful. For example, Molero and Miller (1991) found that two radioactive agonists for cholecystokinin receptors, cholecystokinin and the partial agonist OPE (D-Tyr-Gly-[($Nle^{26,31}$)CCK-26-32]-phenyl ester) demonstrated high affinity binding in bovine gall bladder. However, whereas ^{125}I-OPE binding was sensitive to Gpp(NH)p, high affinity binding of ^{125}I-CCK was not. These data can be interpreted as suggesting that the two agonists activate a different array of G-proteins in this preparation, the binding of some of which are sensitive to Gpp(NH)p (Molero and Miller, 1991). Similar results were obtained by Lallement et al. (1995), who showed that gastrin activation of CCK B receptors in Jurkat cells was sensitive to stable guanyl nucleotides, whereas CCK binding was only slightly affected (Lallement et al., 1995). Another related approach to this technique is the study of selective G-protein activation by agonists with specific antisera for different G-proteins (i.e., Izenwasser and Cote, 1995).

Agonist trafficking of stimulus can be measured as selective efficacy of agonist for receptor/G-protein pairs. An interesting example of this was shown by Meller et al. (1992), who showed that reversed relative efficacy of the dopamine agonists quinpirole and 3-(3-hydroxyphenyl)-N-n-propylpiperidine in the rat anterior pituitary and striatum. These data suggest that these agonists produce differences in coupling to different G-proteins in these two tissues; the reversal of relative potency (i.e., reversed intrinsic efficacy) cannot be accommodated by a strength of signal hypothesis.

There are two important implications of agonist trafficking of receptor stimulus. The first relates to the testing of new drug entities for therapeutic potential. If certain agonists preferentially couple receptors to select G-proteins, then ligands that interfere with receptors and G-protein coupling (i.e., have either positive or negative intrinsic efficacy) may be selective on the basis of the system used to test for drug activity. In this sense, the screen would be for a selected receptor/G-protein pair and not just the receptor. Parenthetically, this has relevance to site-directed mutagenesis studies aimed at defining the binding locus of molecules within receptors. One of the major methods used in these types of studies is the selective antagonism of certain agonists over other agonists. For example, the antagonism of responses to substance-P and septide by RP 67580 differs considerably in COS cells transfected with neurokinin-1 receptors (Pradier et al., 1994). These data can be interpreted as evidence that the two agonists, substance-P and septide, bind to different regions of the neurokinin receptor and that the antagonist does not access both regions equally well. However, an alternative explanation might be that the two agonists use different G- proteins to produce response and that the antagonist discerns the

receptor/G-protein pairs. A second implication of agonist trafficking of receptor stimulus relates to the potential for more selective agonists. It is possible that some agonists produce therapeutic and toxic effects by activation of many G-proteins and that the toxic effects could be reduced by eliminating some of these activations (Gettys et al., 1994).

2. Ligands with protean efficacy. The production of response from a 7TM receptor system consists of two theoretical steps, namely the activation of the receptor (production of activated receptor) and the subsequent coupling of that receptor to the G-protein. In theoretical terms, there is no reason to suppose that all ligands will produce the same effects on receptor activation and receptor/G-protein coupling. In terms of the cubic ternary complex model, the relative magnitudes of the thermodynamic multipliers α and γ may differ. This could occur if the ligand froze the receptor into a unique conformation that had different activating characteristics from the natural spontaneous active conformation. If this were to occur, then the receptor would take on new coupling characteristics with respect to coupling to G-proteins. Under these circumstances, a potentially very interesting experimental condition could exist, because the observed effects of the system would be because of the summation of the spontaneously activated and coupled receptor *and* the agonist-activated G-protein coupled receptor. Unless the two species were identical in terms of their catalytic properties, differences between constitutive activity and agonist-induced activity should be seen. Therefore, depending upon the set point of the receptor system, these compounds may function as positive agonists or inverse agonists, i.e., their efficacy would be protean (Kenakin, 1995c, 1996a). Specifically, the inverse agonism would be observed when the system was predisposed to constitutive activity, and positive agonists when the constitutive activity was low and the response emanates from agonist activation.

Such a ligand may have unique characteristics which, if detected, could in fact offer indirect evidence of a unique agonist-selective active receptor conformation. This type of behavior is modeled in figure 11A. Such simulations suggest that the change from positive to negative efficacy (it should be noted that efficacy is used to describe the combined effect of ligand and receptor as defined by Stephenson (1956) and does not describe the ligand constant intrinsic efficacy) can occur with differences in recetor/G-protein stoichiometric ratios or differences in K_{act} (as in fig. 11A). This latter factor may be effected by changes in ionic environment (Na^+ effect). Another possibility would be changes in K_G that may be approximated by changes in the coupling of the G-protein with activated receptor, which in turn may be effected by GTP availability. Therefore, there may be differences in observed agonist profile in the presence and absence of GTP (fig. 11B).

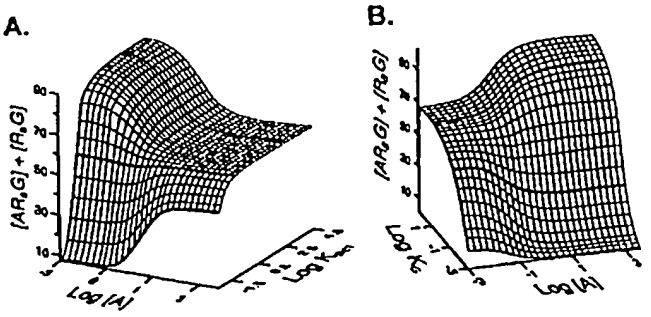


FIG. 11. Protean efficacy. Simulations for a ligand that creates a unique receptor active state which promotes response but is less efficacious than the natural activated state. Ordinates: Basal and ligand-induced response as measured by the quantity of spontaneously coupled receptor [R^*G] plus the ternary species [ARaG]. Data for the observed effects of the ligand in a range of systems. X-axis; Logarithms of molar concentration of ligand. (A) Effect of spontaneous receptor activation (Y-axis = logarithm of Log K_{act}). Under conditions of very low to undetectable spontaneous receptor activation, the ligand is a positive partial agonist. As the amount of highly active spontaneous species ([RaG]) increases (increasing K_{act}), formation of the less efficacious agonist species blocks constitutive activity, and the ligand is an inverse agonist. Systems parameters: β = 10, K_G = 0.1, [R] = [G] = 100. Ligand parameters: α = 100, γ = 0.03, δ = 1. B. Effects of cancellation of the accumulation of ternary complex (i.e., simulated effects of GTP). Y-axis: logarithm of magnitude of K_G. Systems parameters: β = 10, [R] = [G] = 100. Ligand parameters; α = 300, γ = 0.01, δ = 1.

In this type of situation, the agonist could be considered an allosteric effector of the receptor with respect to its coupling to the G-protein. Allosteric effectors for the binding of other ligands such as γ-aminobutyric acid, muscarinic agonists, dopamine and adenosine have been described (i.e., see Birdsall et al., 1995). This idea could be extended to allosteric modification of receptors toward G-proteins to describe changes in the conditional constant γ in the cubic ternary complex model for agonism. There is experimental evidence that this occurs for the adenosine receptor allosteric effector PD 81,723. Specifically, this ligand can be shown to potentiate adenosine agonism by stabilizing receptor/G-protein interaction (Kolias-Baker et al., 1994; Bhattacharya and Linden, 1995).

There are compounds that appear to have complex actions on receptors, being positive agonists in some systems and inverse agonists in others. For example, dichloroisoproterenol is a well known β-adrenergic receptor partial agonist (i.e., see Fleming and Hawkins, 1960); however, in membranes from Sf9 cells overexpressed with β_2-adrenergic receptors, dichloroisoproterenol produced inverse agonism (Chidiac et al., 1994). It is premature to postulate that protean ligands are true pharmacological entities, but if they are found to be so, they may offer a window into agonist-activated receptor states.

3. The molecular nature of efficacy. Efficacy is a molecular property that, under ideal conditions, can be

quantified for the characterization of drug activity (Besse and Furchgott, 1976; Kenakin, 1984, 1985b). What can be seen from the current models of 7TM receptor mechanisms is that efficacy can be thought of as being receptor-related and drug-related. Thus, receptors have efficacy for G-proteins as measured by the equilibrium dissociation constants of the resulting receptor/G-protein complexes (i.e., K_G, fig. 4). The effect of receptor activation on receptor efficacy is given by the conditional term β. These constants quantify the interaction of the receptor protein and G-protein in the membrane. Added to this is the influence of ligands characterized by the effect they have on receptor activation (α term), on G-protein binding (γ term) and the synergy between these two effects (δ term). Theoretically, the delineation of these molecular terms to characterize drugs and receptors would be ideal because the influence of each of these effects on receptor function is different, i.e., there may be different types of efficacy that will result in different types of response in physiological systems.

The previous points have highlighted certain ideas regarding the molecular nature of receptor efficacy. In general, it is reasonable to consider a model of a receptor conformation that exposes various portions of the intracellular loops to G-proteins and that these amino acid sequences serve to activate the G-proteins. In terms of the design of agonist ligands, it is not clear how many active receptor conformations exist in nature. It is well known that proteins are dynamic and that, while at any given instant the protein molecule may be in a distinct conformation, it most likely (at least at physiological temperatures) does not stay there and in fact goes on to explore an 'energy landscape' of different conformations. These fluctuations in conformations may be relaxations toward an equilibrium form or equilibrium fluctuations brought on by ligands (Frauenfelder et al., 1991). An analogy could be made to myoglobin that is thought to exist in two globally distinct macrostates (models of R_i and R_a) and a spectrum of conformational microstates (Frauenfelder et al., 1988). Thus, an energy landscape such as the schematic shown in figure 12A might exist for a given 7TM receptor. Thermal and other energy would control the exploration of the receptor over this energy landscape. In terms of relative populations within this scheme, figure 12B shows the relative population of the quiescent receptor system, most of the species existing in the 'inactive' (R_i) macrostate. A spectrum of potential active microstates exist. It would theoretically be possible for ligands to differentially enrich the relative populations of these various active microstates. Figure 12C shows the relative populations for a hypothetical agonist A1 and figure 12D for another agonist A2. The point would be that these microstates could have different affinities for different G-proteins; thus, agonist selective trafficking of stimulus could occur. At present, these ideas are speculative, but they offer a framework upon which to design experiments to detect agonists that are stimulus-selective.

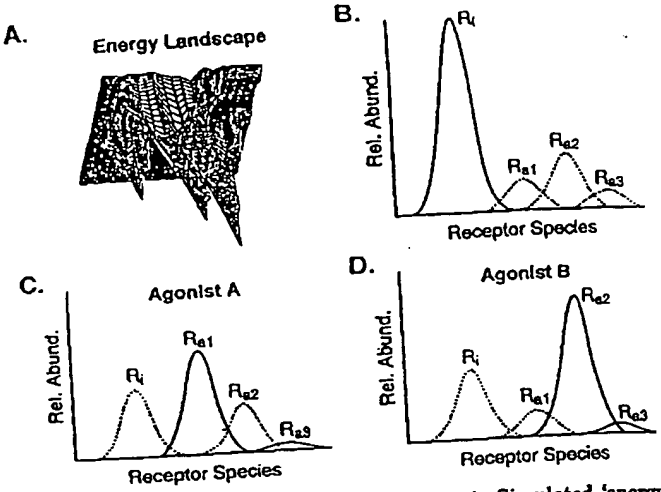


FIG. 12. Energy states of 7TM receptors. A. Simulated 'energy landscape' for 7TM receptors with differing levels of resting energy. The receptor explores this landscape as a function of thermal energy. (B) At any given instant, the relative proportions of receptor states can be illustrated with Boltzman distributions. This shows most of the receptors in the inactive [R_i] state with three putative activated states R_{a1}, R_{a2}, and R_{a3}. C. Theoretical effects of an agonist that enriches active states R_{a1} and R_{a3}. (D) Another agonist enriches active states R_{a2} and R_{a3}. It is postulated that these two different agonists present different arrays of activated receptors to the G-protein complement of the host system. From Kenakin (1996b) with permission.

Parenthetically, the energy landscape idea reconciles Burgen's initial (apparently opposing) views of conformational selection and induction (Burgen, 1966). If a particular receptor conformation was exceedingly rare and a given agonist had a high selective affinity for that conformation, then the agonist would enrich a unique conformation not found in nature in appreciable amounts. Under these circumstances, this would, for all intents and purposes, be conformational induction occurring with a selection mechanism. From this standpoint, conformational selection and induction can be seen to be extremes of the same molecular mechanism of efficacy (Kenakin, 1996b).

VIII. Quantitative Measurements on 7TM Receptors

In general, a large part of pharmacology is the measurement of drug affinity and efficacy; because these are inherently chemical terms specific to the drug and the receptor, they, in turn, can be used to classify receptors. Correct measurement of these drug properties can lead to predictions of therapeutic activity in humans. However, the delineation of systems effects on observed potency first must be achieved.

A. System-Dependent Observed Affinity

The ideal situation is to characterize receptors with neutral antagonists. If a ligand possesses efficacy, then its potency may be *system-dependent* as opposed to only *receptor-dependent*. This is because the components of the system (i.e., receptor level, G-protein composition, level of receptor activation) will affect the ligand receptor binding distribution (i.e., see equation 6). In practical terms, it may be important to detect system-dependent drug activity. This is because, although drugs theoretically are screened from the most simple and stable systems available, they eventually are used in the most complex of systems imaginable, namely the human body under pathological control. Drug discovery screens are designed for robustness and consistency and often will not detect low levels of positive or negative efficacy. However, the resulting discovered compounds are then used under in vivo conditions in which they encounter a spectrum of organs containing different densities of receptor, varying efficiencies of receptor coupling, possibly constitutive receptor foci, and different levels of endogenous agonist tone. In addition, the different membrane milieu for the receptors may contain different mixtures of G-proteins in varying ratios reacting to external hormonal input that possibly would interact (i.e., modulate or potentiate) the receptor signal. For these reasons, it is important to detect system dependence of ligand potency. If such effects are not detected, then the observed potency of a ligand will be assumed to reflect the chemical binding constant, and all differences in potency will be assumed to reflect differences in receptors. This could be dissimulating when receptors are expressed in various host systems, i.e., the potential for artifacts, because of systems effects increases. Moreover, the lack of recognition of system-dependent potency could lead to unexpected differences in activity between screening systems and therapeutic applications in humans.

One way to detect possible system dependence (i.e., efficacy of a ligand) is to measure the variation of repeated estimates of potency. For a true neutral antagonist, the only error associated with measurement would be random measurement error at a given level. If, however, systems effects bias the magnitude of the observed potency, then an added measure of error (that associated with changing relative quantities of components) might be expected in the observed measurement of ligand activity. Figure 13A shows the observed potency of a ligand with positive efficacy in 2000 simulated cell lines, i.e., computer-generated random combinations of [R], [G], and K_{act} for a given receptor/G-protein pair (K_G constant) for a ligand with a constant molecular efficacy ($\alpha = 100$, $\gamma = 1$, $\delta = 1$). As can be seen from this simulation, the observed potency is never less than the equilibrium dissociation constant of the ligand-receptor complex for the inactive receptor (K_A) but often is increased by G-protein coupling, as is commonly observed

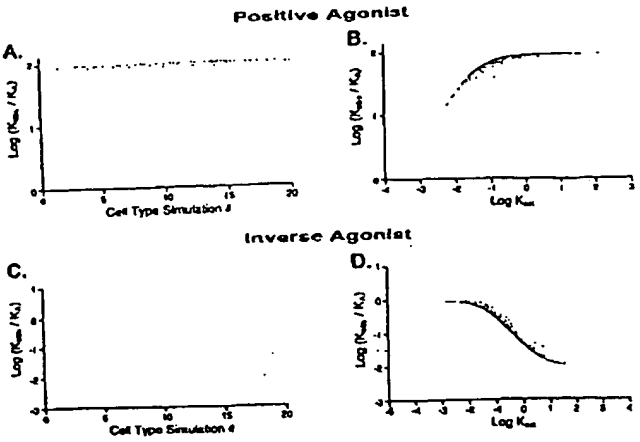


FIG. 13. Simulations of observed affinity for ligands with positive or negative efficacy in 2000 hypothetical cell lines. (A) Observed affinity (as calculated with equation 5) for a positive agonist ($\alpha = 100$, $\gamma = 1$, $\delta = 1$) in a system with varying [R], [G] and K_{act}. It was assumed that the activated receptor/G-protein coupling is favorable ($\beta K_G = 10$). (A) Ordinates: Log (K_{obs}/K_A). Abscissae: Computer simulation number, also a unique randomized configuration of levels of [R], [G] and K_{act} which might correspond to host cell type. (B) Data shown in A grouped as a function of log K_{act}. (C) Same analysis as in A but for an inverse agonist ($\alpha = 0.01$, $\gamma = 1$, $\delta = 1$). (D) Data grouped as a function of log K_{act}.

with agonists. Figure 13B shows the correlation of the observed potency with the working constant for receptor activation (i.e., K_{act}).

An identical analysis was done for an inverse agonist of $\alpha = 0.01$, $\gamma = 1$, $\delta = 1$ (fig. 13C and D), where it can be seen that the observed potency is *decreased* by receptor/G-protein coupling effects. However, the observed potency is never above K_A. These simulations show the expected variation of observed potency of ligands with efficacy (either positive or negative). What should be stressed is that the estimations in each particular cell system are correct for that particular milieu of receptor and G-protein. Moreover, it can be shown that the magnitude of the efficacy is directly proportional to the variation in observed potency (i.e., weak agonists will vary less than strong agonists). This can readily be inferred from equation 6 and the placement of ligand constants α, γ, and δ. Therefore, one practical test of system dependence is the presence of an inordinately high error for the mean measurement of affinity. If the measured affinity of a given ligand is found to be variable with repeated testing (i.e., in a series of transient expression systems or on the receptor when it is expressed in different cellular hosts), then this might imply that the ligand possesses efficacy that causes different interactions of the receptor with G-proteins in different systems.

B. The Manipulation of Receptor Systems

Just as it is possible to experimentally manipulate some aspects of signal strength from natural receptors

(Kenakin, 1984), there are techniques becoming available to modify the strength of recombinant receptor expression signals; i.e., receptor gene induction, increased expression (Collins et al., 1991; Charness et al., 1983, 1986, 1993; Gianoulakis, 1989; Hu and Hoffman, 1993). For example, steroid hormones are well known to affect gene transcription for some receptors (Collins et al., 1989; Davies and Lefkowitz, 1984; Emorine et al., 1987). In contrast, estrogen reduces the number of α_2-adrenergic receptors in the high affinity state in rat hypothalamus (Karkanias and Etgen, 1994). Short-term exposure to β-adrenergic receptor agonists or cAMP analogues can produce elevations of β_2-adrenergic receptor mRNA (Collins et al., 1988). Chronic treatment of SH-SY5Y cells with low efficacy μ-opioid agonists increases the abundance of G-proteins (Ammer and Schulz, 1993). Cotransfection of vectors containing cDNA for the human β_2-adrenergic receptor and for dihydrofolate reductase led to a control of expression levels for the receptor by stepwise increases in methotrexate concentration in the culture medium of CHO cells (Lohse, 1992). This technique, however, was cell-specific, as identical conditions in HeLa cells led to cell death. In cyclosporin A-induced hypertensive rats, increased gene expression for angiotensin type II receptors has been reported (Iwai et al., 1993). Another means of affecting receptor transcription has been shown for 5-HT type 2 receptors, where chronic treatment with antagonists led to reductions in both receptor and mRNA (Toth and Shenk, 1994)

In general, there is an increasing body of experimental evidence to indicate that, by the use of various promoters and plasmids, the control of receptor stoichiometry in host cell systems can be achieved. A strategy using adenovirus-mediated gene transfer has been used with several mammalian genes (Mulligan, 1993), including the expression of thyrotropin-releasing hormone receptors in several cell lines (Falck-Pedersen et al., 1994). Receptor expression can be induced, as was the case for α_1-adrenergic receptor transfected with isopropyl-β-D-thiogalactoside-inducible vectors in SK-N-MC cells (Esbenshade et al., 1995a, b). One of the most promising areas in this technology is the co-expression of receptors and G-proteins to create 7TM receptor systems. For example, cotransfection of Rat-1 fibroblasts with cDNA for α_{2A}-adrenergic receptors and cDNA for G_{o1} produces activation of this foreign G-protein in the transfected cell (Grassie and Milligan, 1995). Similarly, the cotransfection of $G_{\alpha s}$-subunit with secretin receptors greatly increased the amount of high affinity ternary complex coupling seen with ^{125}I-secretin (Ishihara et al., 1991). Co-expression of G-protein with receptors also has been carried out successfully with 5-HT_{1c} receptors (Quick et al., 1994), 5-HT_{1A} receptors (Butkerait et al., 1995), somatostatin-3 receptors (Law et al., 1994), α_2-adrenergic receptors (Coupry et al., 1992) and opioid receptors (Tsu et al., 1995).

The measurement of drug affinity and efficacy uses techniques unique to two broad disciplines, radioligand binding and those specific for functional systems. It is worth considering these separately.

C. Radioligand Binding

Binding studies offer a unique perspective on drug receptor interaction, in that theoretically the complex between the ligand and the receptor can be studied directly. The most simple model upon which all analyses initially are based is the Langmuir adsorption isotherm (Langmuir, 1916). The basic premise of this model is that molecules bind to an inert surface and that the equilibrium dissociation constant of the molecule/surface complex is a chemical term dependent only upon the nature of the two entities bound to each other. In fact, Langmuir derived his equation in terms of the actual area bound and not bound by an adsorbent material onto a surface. If this can be shown to be the case for a drug and receptor, then this chemical term assumes great importance, as it can be used to characterize that receptor in any tissue in which it resides. There are two major areas for error in the classification of receptors with ligand binding. One is the introduction of systems effects because of undetected ligand efficacy, and the second is heterogeneity of receptor populations.

Tests for Langmurian kinetics generally ask the question, "Is the observed binding consistent with the interaction of a ligand with a single stable receptor population?" The two windows into ligand binding behavior are by saturation binding of a radioligand and by inhibition binding of a fixed amount of radioligand by a nonradioactive ligand. The first expectation of Langmurian kinetics is that the binding curves for both types of experiment be monophasic and have a Hill coefficient not significantly different from unity. There have been numerous publications on mechanisms and nuances that produce complex behavior of binding curves (i.e., see Limbird, 1996; Swillens et al., 1995) that need not be reiterated here. Interestingly, the very nature of transfected cellular expression systems in which the receptor levels may be high can lead to artifacts with the use of standard binding models that assume that the concentration of radioligand is not altered by receptor binding (Swillens, 1995).

In general, two conditions can lead to complex binding curves or nonadherence to Langmurian kinetics: the presence of efficacy in a ligand that interacts with the receptor and one or more G-proteins, and/or the presence of a mixture of binding sites, either multiple stable binding sites (as in splice variants of a receptor) or multiple pre-existing coupling states. The latter condition (multiple coupling states) requires that the ligand have efficacy before differences in observable binding can be seen.

1. Saturation binding experiments. The study of expressed receptors in surrogate cell lines theoretically

should have the advantage that a single protein is expressed; therefore, a subtle presence of a mixture of receptor subtypes will not complicate the analysis. Under these circumstances, a monophasic saturation curve representing ligand binding to a single population of receptors is expected (in the absence of ligand efficacy). However, the transfection of cells with genomic receptor clones may not always lead to the expression of pure populations of receptors. For example, a genomic clone for the bradykinin receptor isolated from a mouse cosmid library leads to the expression of an apparently mixed population of B_1 (30%) and B_2 (70%) bradykinin receptors in COS-7 cells (McIntyre et al., 1993). While this appears not to be due to RNA splicing, it is not clear whether the mixed population is the result of incomplete post-translational modification or RNA editing. An alternative possibility would be the promiscuous coupling of the expressed receptor with different G-proteins.

This raises a practical point in receptor classification, namely the differentiation between heterogeneous receptors, receptor binding states (with G-proteins) and heterogeneous binding *sites* on the same receptor molecule. The technique of site-directed mutagenesis has opened a new era for the study of structure-activity relationships. Thus, the effects of genetically induced single-point mutations in receptors on the affinity of different ligands can be used to delineate separate loci of binding of different molecules on the same receptor. Notable examples of this approach are the different binding sites for peptides and nonpeptides on peptide receptors (i.e., see Perlman et al., 1995; Gether et al., 1993, 1994, 1995; Xue et al., 1994; Schwartz, 1994). An important tool in this endeavor is the saturation binding curve of different ligands because, in theory, these allow the ability to *count* binding sites as well as determine affinity. Therefore, a simple test for the assumption of different binding loci on the same receptor is the criterion that the *number* of sites for both selective ligands be the same (fig. 14A). Failure to demonstrate this leads to conclusions of different ligand-induced receptor conformations interacting with different G-proteins to produce different binding species in the membrane (fig. 14B). Especially in expression systems in which the stoichiometry of receptor to G-proteins may encourage promiscuous receptor coupling, the potential for multiple agonist activated ternary complexes with different G-proteins should be considered. If these agonist-selective species are formed, then separate structure-activity relationships could be observed for them, i.e., agonist selective functional antagonism, or binding affinities, could be observed. This possibility should be eliminated before consideration of specific receptor regions for binding.

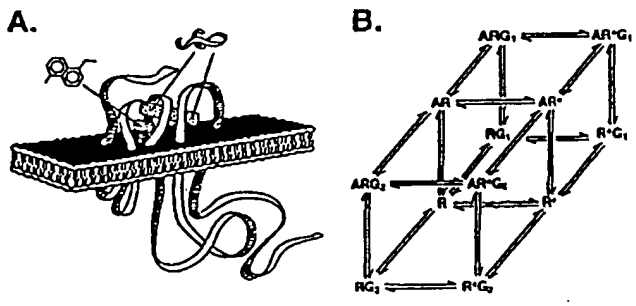


FIG. 14. Two potential views of selective agonist activation of 7TM receptors. (A) Two ligands bind to two separate allotopic sites on the receptor. (B) The receptor can form two separate complexes with two different G-proteins. The agonists differentially direct the receptor toward each G-protein, respectively.

The saturation maximal asymptote for radioligand binding can be useful to differentiate selective sites from selective confirmational species. However, care must be taken in the use of B_{max} values because of their inherent inaccuracy (Klotz, 1982; Klotz and Hunston, 1984). Usually, the maximal asymptote of a saturation binding curve is difficult to define with real data, and the shape of the saturation curve is used to estimate its value. However, in the case of agonists being tested in a possibly overexpressed receptor systems, high affinity binding measures the G-protein coupled receptor and the B_{max} value depends upon the ratio of receptor to G-protein available for coupling. In highly expressed systems, the receptor densities may outstrip the G-protein coupling capability, leading to a relatively small population of high affinity binding sites when compared with total receptor binding. Under these circumstances, the magnitude of the ratios between the high and low affinity binding sites could greatly affect the estimated B_{max} by saturation analysis. For example, figure 15A shows the saturation binding curve for a hypothetical agonist with efficacy values $\alpha = 10$, $\gamma = 1000$, $\delta = 1$ in a system of limited G-protein ([R]/[G] = 3.3). The delineation between the apparent high and low affinity sites for this agonist are clear, and the B_{max} estimate from data points is relatively immune to the size of the dataset. Figure 15A also shows the saturation curve for another agonist with a less clear delineation between the apparent high and low affinity sites ($\alpha = 100$, $\gamma = 100$, $\delta = 1$). For this agonist, the estimated B_{max} value depends very much on the size of the dataset in that, the higher the concentrations of radioligand that are used, the further up the secondary phase of the curve goes the binding (see fig. 15C). This is not a relevant issue for the agonist with the more clearly delineated biphasic binding (fig. 15B); however, the dependence of the size of the dataset for the two agonists can cause an increase in the apparent B_{max} (fig. 15D). It can be seen from these simulations that complex saturation curves should be analyzed carefully before conclusions regarding the relative size of receptor populations are made. The conclusion drawn from different B_{max} values is far reaching in an expression system because it implies the production of either two different expressed species, or that the receptor is

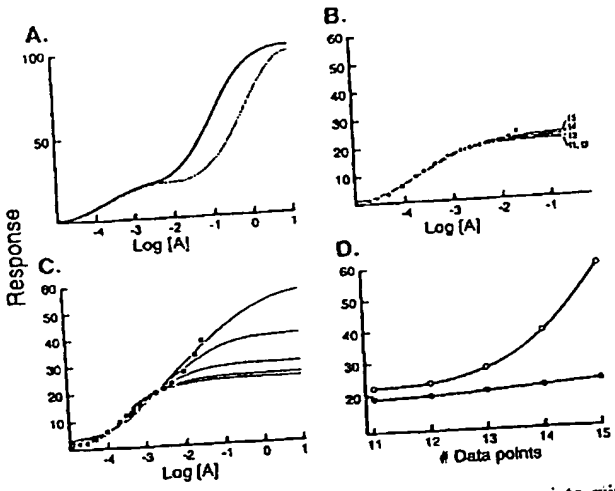


FIG. 15. Total saturation binding curves for two agonists with varying intrinsic efficacy to promote formation of the ternary complex (i.e., observed as high affinity binding). Ordinates; Production of [ARaG] and [AR] by the agonist. Abscissae; Logarithms of molar concentration of agonists. (A) Complete saturation curves for two agonists of varying efficacy; Solid line $\alpha = 100$, $\gamma = 100$, $\delta = 1$ and dotted line $\alpha = 10$, $\gamma = 1000$, $\delta = 1$ in a G-protein limited expression system ($\beta = 10$, [R]=100, [G]= 20, $K_G = 0.1$). (B) Statistically fit saturation curves for datasets of varying size for dotted line agonist in A. Numbers next to curves represent the number of datapoints used for the fit. (C) As for B but for solid line agonist in A. For this ligand, the size of the dataset severely affects the estimated B_{max}. (D) The estimated B_{max} values for the two agonists (dotted line agonist represented by filled circles) as a function of the number of datapoints used for the fit.

coupling to at least two different G-proteins. Both of these conclusions greatly affect how the receptor data from such a system is interpreted; therefore, the veracity of different B_{max} values becomes paramount.

In view of data clearly showing that receptors can be promiscuous with respect to the G-proteins with which they interact and also, that the expression level of receptors can dramatically alter G-protein coupling, the possibility of aberrant receptor/G-protein coupling must be considered in all transfection experiments. It is difficult to gauge the 'correct' physiological receptor/G-protein interaction because mass stoichiometry has no meaning within the constraints of the membrane. As discussed by Neubig (1994), cytoskeletal elements in the membrane and other factors can severely limit access of receptors to G-proteins. Therefore, a 100-fold greater bulk amount of G-protein over receptor is meaningless in terms of the actual G-protein accessed by the receptor in a membrane. There may, in fact, exist microdomains of receptors and G-proteins in cell membranes with asymetrically distributed receptors or G-proteins (Van Zastrow et al., 1993; Keefer et al., 1994; Neer, 1994). There are examples of situations in which the relative quantity of high affinity binding sites is limited by the availability of G-protein in the surrogate cell system. For example, as discussed earlier, in COS cells transfected with cDNA encoding the rat secretin receptor, only 1.7% of the sites binding ^{125}I-secretin were of high affinity. However, cotransfection of G_s protein increased the relative quantity of high affinity binding to 15% of the total (Ishihara et al., 1991). It should not be assumed that receptors have the same access to G-proteins in a membrane and will universally couple to those G-proteins (i.e., converse to receptor promiscuity). For example, α_{2B} and α_{2A} adrenergic receptors expressed transiently in human choriocarcinoma (JEG-3) cells have access to the same complement of G-proteins yet selectively couple to different effectors (Pepperl and Regan, 1993). Similar results were obtained by Hughes et al. (1986), who showed that, although muscarinic receptors normally interact with G_i, they do not do so in 1321N1 astrocytoma cells, even though a functionally active G_i can be shown with adenosine receptors.

2. Inhibition experiments. Another standard binding assay used to classify ligands and receptors is the quantitative inhibition of the binding of a fixed amount of radioligand to a receptor by a nonradioactive ligand. This procedure is essentially based on the mathematical model for simple competitive antagonism first presented by Gaddum (1937):

$$\frac{[A \cdot R]}{[R]} = \frac{[A]/K_A}{[A]/K_A + [B]/K_B + 1} \qquad [7]$$

in which the concentration of the ligand being blocked (i.e., radioligand in binding studies) is denoted by A, the equilibrium dissociation constant of the complex between A and the receptor denoted K_A, the blocking ligand denoted B, and the equilibrium dissociation constant of the complex between the blocking drug and receptor denoted by K_B. From this model comes Schild analysis for functional studies (Arunlakshana and Schild, 1959; vide infra) and the models for calculating the equilibrium dissociation constants for ligand/receptor complexes in binding studies (denoted K_i). Rearrangement of equation 7 can give a very useful, experimentally accessible relationship between the concentration of radioligand and the concentration of nonradioactive ligand required to block it. Thus, it can be shown that the concentration of ligand that reduces the binding of a given concentration of radioligand by 50% (denoted as the IC_{50}) is related to the concentration of radioligand by the following relationship:

$$\frac{IC_{50}}{K_i} = \frac{[A^*]}{K_d} + 1 \qquad [8]$$

in which K_d denotes the equilibrium dissociation constant of the radioligand-receptor complex obtained from saturation binding studies. The equation often is referred to as the Cheng-Prusoff relationship (Cheng and Prusoff, 1973). The important point to note about this equation is that the relationship between the IC_{50} and

the concentration of radioligand is a straight line. Therefore, even if the K_d and/or the K_i for the drugs are not known, deviation from the model can be detected by observing the relationship between the IC_{50} and [A*]. This then becomes a very simple test for receptor and/or binding-site heterogeneity along various regions of the saturation binding curve. If the relationship between radioligand concentration and IC_{50} is not linear, this would imply that something other than, or in addition to, binding to a single static population of sites was occurring.

3. Binding and receptor biochemistry. The ability to label and track receptor protein has led to many techniques for the study of receptor structure, state and, relevant to the discussion of receptor classification, coupling to G-protein. For examples, receptors can be solubilized and immunoprecipitated with antisera for G-proteins (i.e., Law et al., 1991; Chatterjee et al., 1993; Gurdal et al., 1995) or receptors (Matesic et al., 1989, 1991). Similarly, antisera directed the C-terminal region of G-proteins have been used to disrupt agonist activation of receptors and/or high affinity binding (McKenzie and Milligan, 1990; Milligan et al., 1995b). Antisera directed to the N-terminal end of G-proteins have been used to co-immunoprecipitate receptors with G-proteins (Okuma and Reisine, 1992; Law et al., 1991). The cross-linking of receptor proteins to G-proteins also has enabled the study of receptor/G-protein interaction (i.e., Kermode et al., 1992). Another approach is to observe agonist-induced incorporation of [α-^{32}P]AA-GTP (a GTP analogue azidoanilidido[α^{32}P]GTP) into various G-proteins (Prather et al., 1994; Palmer et al., 1995). A novel method to study the activation of G-proteins by agonist-stimulated receptors is the observation of the half-time for degradation of G-protein in the presence and absence of agonist (Wise et al., 1995). Agonist affinity columns have been used to purify receptor/G-protein complexes as well (Munshi and Linden, 1989; Munshi et al., 1991). Another method of purification is with a biotinylated radioactive agonist for receptor binding followed by separation over a strepavidin affinity column (Eppler et al., 1992; Luthin et al., 1993).

Other methods use pertussis toxin (PTX)- or cholera toxin (CTX)-catalyzed ribosylation of G_α subunits. Receptor and agonist activation of PTX-sensitive G-proteins can be detected by taking advantage of the fact that PTX preferentially catalyzes ADP ribosylation of the G-protein heterotrimer (Milligan, 1987). Thus, agonist activation of a particular G-protein, if PTX-sensitive, will be diminished (Brass et al., 1988). In contrast, CTX interacts preferentially with the free α-subunits (Milligan and McKenzie, 1988); thus, agonist and receptor activation of a CTX-sensitive G-protein would increase adenosine diphosphate ribosylation (i.e., Milligan and McKenzie, 1988; Klinz and Costa, 1989). Theoretically, these methods may furnish a direct way to detect agonist trafficking of receptor stimulus if it could be shown that different agonists produce different patterns of ternary complexation.

D. Functional Studies in Receptor Classification

Early receptor classification relied completely on functional experimentation, and a great deal of pharmacology was concerned with the cancellation of tissue effects (usually through the null method). The introduction of receptor binding technologies has added another dimension to receptor pharmacology, and new insights into drug-receptor interaction were obtained. Now, the availability of new technologies and the advancement of biochemical techniques for receptor study have expanded the realm of receptor research back into functional receptor experiments beyond isolated tissues. It is now possible, by a variety of mechanisms, to study agonist efficacy in membrane receptor preparations and in cell culture (vide infra). This allows the considerable theoretical advantages of functional systems to be exploited. Many of these advantages (and disadvantages) are common to all methods of functional experimentation.

Before these are discussed specifically, there are several tacit assumptions that should be considered when transfected receptors and host cells are assembled for functional assays. The fact that the correct signal transduction apparatus may exist in the membrane of some cells still does not ensure that the biochemical mechanisms for transforming the stimulus to a cellular response are present as well. For example, whereas transfection of human dopamine D_3 receptor cDNA into CHO-K1, SK-N-MC, or CCL1.3 cells produces high affinity radioligand binding (with sensitivity to GTP analogues, indicating G-protein coupling), no effects on cAMP accumulation, inositol phosphate production or arachidonic acid release was observed with dopamine agonists (MacKenzie et al., 1994). Similarly, whereas β_1-, β_2- and β_3-adrenergic receptors co-exist in hamster brown fat cells, it appears that only the β_3-adrenergic receptors participate in thermogenesis (Zhao et al., 1994). It cannot be assumed that the activation of a receptor system to elevate the cytosolic level of a second-messenger automatically will make the intracellular second-messenger accessible to all parts of the cell machinery. For example. both dopamine and isoproterenol elevate intracellular cAMP in transfected kidney CV_1 cells. However, although the dopaminergic elevation of cAMP produces a translocation of transfected progesterone receptors from the cytoplasm to the nucleus, isoproterenol was *not* able to produce this same effect (Power et al., 1991). The need for other systems for the production of a functional response also can be very important. For example, transfection of the rat neurotensin receptor into 293 cells was insufficient to produce a functional system for producing cyclic guanosine monophosphate synthesis until nitric oxide synthetase cDNA was subcloned into the expression vector (Slusher et al., 1994).

The cell type used for expression could be very important for the functional study of receptors. For example, whereas transfection of the isoform for the somatostatin receptor mSSTR2A into CHO-K1 cells showed somatostatin-mediated inhibition of adenylate cyclase (Strnad et al., 1993; Vanetti et al., 1993b), stable transfection of the same receptor in CHO-DG44 cells or transient transfection into COS-1 or HEK 293 cells failed to show this same functional effect (Rens-Domiano et al., 1992; Law et al., 1993). These data may partially be explained by the fact that $G_{\alpha i1}$ protein (Tallent and Reisine, 1992), found to be necessary for somatostatin receptor function in AtT-20 cells, is present in CHO-K1 cells (Gerhardt and Neubig, 1991), but not in CHO-DG44 cells (Rens-Domiano et al., 1992).

1. New technologies for cellular systems. One obvious advantage of functional experiments is the increased sensitivity obtained by using the biochemical cascades in cells to amplify low levels of stimulus. The sequential relationship between saturable biochemical reactions in cells leads to amplification of minute membrane signals. For example, carbachol in CHO cells shows a 2.6-fold amplification between receptor occupancy and phosphoinositide hydrolysis and a further 88-fold amplification from PI hydrolysis to nitric oxide release (Wang et al., 1994).

Exquisite selectivity can be gained with functional preparations because many of them yield strong signals (maximal responses) with occupation of very small fractions of the receptor population. Figure 16 shows a schematic diagram depicting a typical 7TM receptor biochemical cytosolic cascade. Technology now has allowed viewing of the consequences of a drug-receptor interaction at various stages along this pathway. Denoting the interaction of the agonist and the 7TM receptor as reaction 1, the first consequence is the activation of G-proteins in the cell membrane (step 2). This can be viewed several ways, including as activity of the intrinsic GTPase activity of the G-protein and binding of radioactive GTP analogues to G-proteins (i.e., Freissmuth et al., 1991; Traynor and Nahorski, 1995; Odagaki and Fuxe, 1995; Thomas et al., 1995). Another first step in this cascade is the direct activation of ion channels with $\beta\gamma$ subunits of G-proteins resulting from G-protein activation (Jelsma and Axelrod, 1987; Okabe et al., 1990).

Further on in the sequence is the production of second-messengers such as cAMP or IP_3 (step 3). Electrical readouts of response from receptors expressed in oocytes is a well established technology (for typical examples see Wank et al., 1992; Racke et al., 1993; Kubo et al., 1986; Yu et al., 1991; Sundelin et al., 1992; Yakel et al., 1993; Maricq et al., 1991; McEachern et al., 1991; Minami et al., 1993). Interesting variants on the use of oocytes for response measurements is the co-injection of antisera to identify which G-proteins are activated by agonists (McFadzean et al., 1989; Harris-Warrick et al., 1988; Jones et al., 1994). New approaches coupled with molecular biology have extended these approaches to yield a new collection of functional assays for the study of efficacy in transfected cells. A rapidly expanding technology is in the field of reporters for cytosolic second-messengers. Thus, the next step in the cascade can be viewed with reporters for receptor-active cytosolic products. Basically, there are two types of reporters; reporter genes and reporter proteins. The former produce a readout of receptor activation by introducing a gene that is affected by the second-messenger. Under these circumstances, receptor activation leads to increased transcription and expression, and the magnitude of this secondary response is quantified some hours later. A second type of reporter system is the introduction (now by genetic means) of reporter proteins that signal the elevation of second-messengers directly in the cytosol.

The use of fusion genes is increasing as a means of assessing gene expression and thus, indirectly, assessing increases in cytosolic second-messengers. With this method, a promoter activator binding site or enhancer sequence is attached to a gene directing synthesis of a reporter molecule. The quantity of the reporter molecule in the cytosol thus becomes an indicator of the avidity of gene expression which, in turn, indicates the level of second-messenger present in the cytosol during expression. For example, elevation of intracellular cAMP or calcium (by calmodulin kinase) results in phosphorylation, and subsequent activation of the transcription factor cAMP response element binding protein (Gonzalez and Montiminy, 1989; Yamamoto et al., 1988). The level of the reporter can be assayed either from the cell lysate or culture medium (for secreted reporter proteins). This latter factor is relevant in terms of whether the response is monitored in real-time or stop-time techniques.

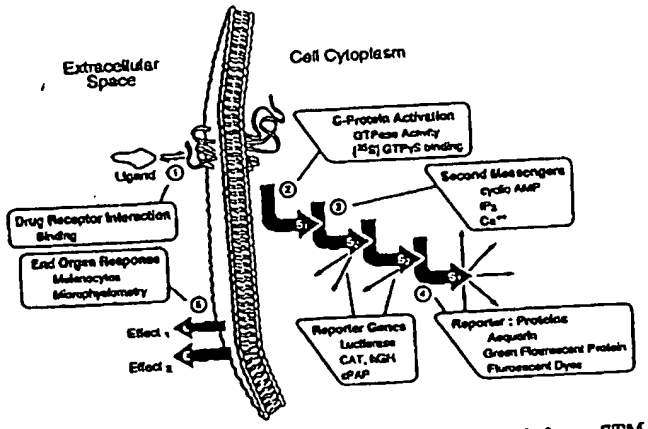


FIG. 16. Stimulus-response cascades in the cytosol for a 7TM receptor. Steps refer to the binding of a ligand to the receptor (1), the activation of a G-protein (2), the production of a second-messenger (3), the interaction of the second-messenger with cytosolic mechanisms (and detection with a reporter system (4)), and the complex observable end-organ response (5).

Reporter assays can be radioactive or nonradioactive. The firefly luciferase reporter system uses a bioluminescence reaction catalyzed by luciferase and luciferin. The intensity of the light observed is an indirect estimate of the efficiency of transcription of the luciferase gene. This can be particularly useful if a reporter cell line is established into which receptors could be transfected. For example, a reporter cell line containing the reporter *Photinus pyralis* luciferase gene (De Wet et al., 1987) under the transcriptional control of either a regulatory sequence responsive to cAMP (Himmler et al., 1993) or IP_3/diacylglycerol (Weyer et al., 1993) has been developed and used to study the function of transfected 5-HT_2 receptors (Weyer et al., 1993), NK1, NK2, NK3 receptors (Weyer et al., 1993; Stratowa et al., 1995), dopamine D_1 and D_5 receptors (Himmler et al., 1993), and muscarinic m1 and m4 receptors (Migeon and Nathanson, 1994).

Another nonradioactive reporter assay uses β-galactosidase (coded from the *E. coli* lac Z gene fused to a cAMP responsive element); the level of this reporter is assayed colorimetrically or fluorometrically from photoactive substrates. This method has been used to assay the function of receptors linked to G_s and G_q (Chen et al., 1995). Similarly, α_{1B}-adrenergic, m4 muscarinic, NK1 neurokinin and trkA neutrotrophin receptors transfected into NIH 3T3 cells yielded functional responses with this reporter (Messier et al., 1995). Some reporter assays use radioisotopes as in the chloramphenicol acetyltransferase assay. In this method, radioactive chloramphenicol is incubated with cells for a period of time after which the acetylated and nonacetylated forms of the substrate are measured. This approach has been used to study α_2-adrenergic receptor function in JEG-3 cells (Pepperl and Regan, 1993). A similar radioactive reporter assay uses human growth hormone (Selden et al., 1986).

Some reporter assays use secreted products and thus can be used for real-time assays. For example, a gene that encodes a truncated secreted human placental alkaline phosphatase (SPAP) can be used for colorimetric or bioluminescent assays (Berger et al., 1988). The levels of the secreted SPAP are directly proportional to SPAP mRNA and protein (Cullen and Malim, 1992). The secretion aspect of this assay is advantageous in that the cells are not disturbed during the assay; therefore, results can be obtained in real time, the background signal is nearly absent and the assays can be automated.

Reporter assays also have been developed for in vivo use. Under these conditions, reporter proteins can be encoded in transfected reporter genes and the cells made to express them in situ. Thus, green fluoresent protein (Chalfie et al., 1994) can be expressed in cells and used to monitor gene expression. The availability of luciferase substrates capable of crossing the cell membrane (i.e., caged luciforin) theoretically allows the use of the luciferase reporter assay in real time (Bronstein et al., 1994; Yang and Thomason, 1993).

As well as reporter genes for the measurement of cellular response, reporter proteins have been used. These molecules directly signal the level of cytosolic messengers such as Ca^{2+} (i.e., aequorin, Fura dyes) or other ions (Tsien, 1989). These can be introduced into the cells (i.e., microinjection of aequorin into oocytes, Giladi and Spindel, 1991) or co-expressed in the cell. The use of green fluorescent protein may be extremely versatile. This bioluminescent protein, when excited with light at 396 nm, will emit light at 508 nm. No preparation is required in that the cells need only to be illuminated with light and the resulting luminescence measured. The level of luminescence is directly proportional to the level of green fluorescent protein; thus, it can be used as a reporter for any inducible promoter (i.e., cAMP or diacylglycerol reporter genes).

There also are functional assays recording more complex responses from cells. For example, the measurement of cellular metabolism by microphysiometry allows a broad range of cellular responses to be measured. The concept relates to the fact that the rate of cellular metabolism is directly linked to the hydrogen ion extrusion by the cell, and this can be measured as the pH in the medium surrounding the cell. This is an extremely valuable technique because it can be used for virtually any cell type that can be sustained in culture (McConnell et al., 1992; Hafeman et al., 1988). Figure 17A shows the effects of human calcitonin on type 2 human calcitonin

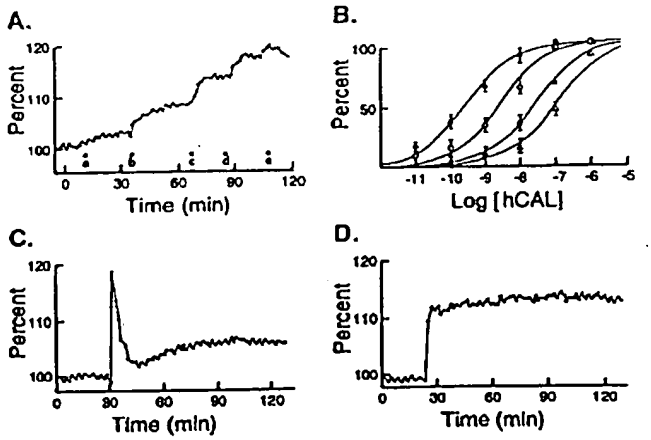


FIG. 17. Cytosensor microphysiometer responses for HEK 293 cells transfected with human calcitonin receptor type 2. (A) Cumulative dose-response curve for human calcitonin. Ordinates: percentage increase of basal cellular hydrogen ion secretion. Abscissae: Time in min. a = 10 pM, b = 100 pm, c = 1 nM, d = 10 nM human calcitonin, and e = 100 nM salmon calcitonin. (B) Schild analysis for human calcitonin responses. Responses in the absence (filled circles, $n = 12$) and presence of various concentrations of peptide calcitonin antagonist AC512 (Watson et al., 1995) 10 nM (circles, $n = 12$), 100 nM (filled squares, $n = 14$), and 300 nM (open triangles ($n = 12$). (C) Cytosensor response to 1 nM human calcitonin in a high expression HEK cell line (28,000 fmol/mg protein receptors). (D) Cytosensor response to 1 nM human calcitonin in a low expression HEK cell line (65 fmol/mg protein).

receptors expressed in HEK 293 cells. As can be seen from this figure, a cumulative concentration-response curve can be obtained from this system. Figure 17B shows the dextral displacement of human calcitonin dose-response curves by the peptide calcitonin antagonist AC512 (Watson et al., 1995). The resulting Schild analysis yields a linear Schild regression with a slope not different from unity. Figure 17 C and D show the effects of receptor expression level on steady-state response and underscore the value of real-time data and the potential problems with high expression levels. Although a low receptor density yields a monotonic response pattern with a sustained steady-state response (Clone 134–4-7: fig. 17D, 65 fmol/mg protein), the high receptor expression clone (Clone 134–2-23, fig. 17C, 28,000 fmol/mg protein) shows a triphasic response. This is consistent with promiscuous coupling of the calcitonin receptor to different G-proteins (Horne et al., 1994) to produce conflicting signals and complex responses. In general, high receptor expression levels may not be desirable for functional experiments.

There are alternative methods to detect ligand intrinsic efficacy biochemically or in cell lines. For example, *Xenopus laevis* melanocyte cell lines can be used to study the recombinant activity of G-protein receptors that modulate either cAMP or phosphoinositide production. Specifically, the dispersion or aggregation of pigment-containing melanosomes is affected by second-messengers and thus can be traced by observing light transmission at 620 nm (McClintock et al., 1993; Karne et al., 1993; Graminski et al., 1993; Potenze et al., 1992, 1994; Lerner, 1994).

The central dogma regarding functional receptor pharmacology is the idea that, if the end organ response is the result of a succession of saturable biochemical functions, then an amplification of the original signal is produced. In terms of drug development, this may be an advantage because an extremely weak initial signal may become measurable if viewed further on down the series of reactions. An example of this is the extremely weak activity of the β-adrenergic receptor partial agonist prenalterol on adenylate cyclase and the powerful end-organ cardiac response (Hedberg et al., 1982). Another example is the rate of myocardial relaxation of isolated cardiac preparations. In the guinea pig atrium, the dose-response curve for increased rates of myocardial relaxation can be detected at concentrations of externally applied dibutryl cAMP, which do not produce any other physiological effect on the preparation (fig. 18; Kenakin et al., 1991). This carries over into the study of weak partial agonists as well. Thus, while the β-adrenergic receptor partial agonist prenalterol produces nearly negligible inotropic activity in guinea pig left atria, a powerful myocardial relaxant effect can be observed. This illustrates the idea that, by selection of the biochemical readout from the cytosolic cascade, measures of efficacy can be obtained (Kenakin et al., 1991).

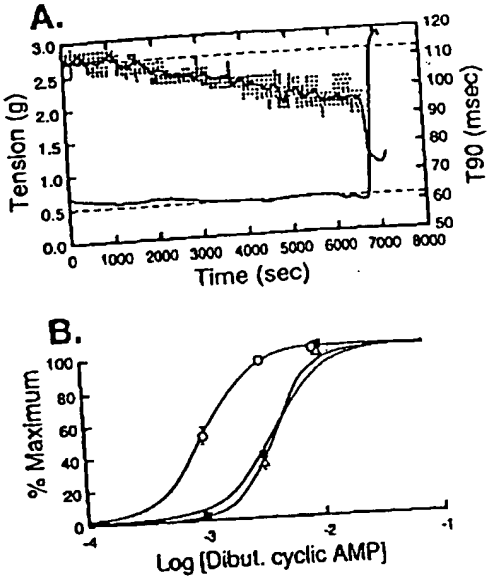


FIG. 18. Effects of dibutryl cAMP on guinea pig atrial function. (A) Temporal response to 1 mM dibutryl cAMP. Upper tracing quantifies the T_{90} (time to 90% relaxation after twitch contraction in msec); lower tracing shows lack of inotropic response (in g tension) in the same preparation. (B) Dose-response curves for myocardial relaxation (open circles) and inotropy measured at peak response (filled circles) and at 90 min (open triangles). Data expressed as the percentage of the maximal response to 10 μm forskolin in the same preparations. From Kenakin et al. (1991).

Reporter systems can be particularly sensitive for the detection of agonism because the second-messenger produced, in the case of the luciferase assay, goes on to initiate a series of reactions, including transcription, that result in the expression of luciferase. The fact that sequential biochemical reactions are involved leads to considerable amplification.

On the other end of the spectrum, increased sensitivity also can bring increased complexity, leading to complicating cross-reactions that may obscure the signal. With the measurement of end organ responses, or even responses that are processed by cytosolic reactions, can come a loss in fidelity of the signal.

Another major advantage of functional studies is that many of the techniques yield data in real time as opposed to the use of arbitrary windows of reaction time (although there are binding techniques such as fluorescence cytometry that allow binding studies in real time as well, i.e., Fay et al., 1991; Heithier et al., 1994; Neubig and Sklar, 1993). Both of these strengths can be especially important in functional studies of cell lines transfected with receptors because of the phenomenon of overexpression. If there is altered stoichiometry between the receptors and G-proteins, then aberrant coupling may produce conflicting physiological responses. For example, different levels of α_{2A}-adrenergic receptor expression levels in CHO cells produce different re-

sponses to the α_2-adrenergic receptor agonist UK-14304. At a receptor level of 1 pmol/mg protein, a dose-dependent inhibition of adenylate cyclase activity is obtained, whereas at 5 pmol/mg protein receptor, a biphasic inhibition followed by stimulation is observed (Eason et al., 1992). In this case, further analysis indicated that the receptor coupled to G_i at low levels and both G_i and G_s at higher levels. When these complex responses occur in expressed systems, unless the time course for activation of the pleiotropic pathways is identical, a corresponding complex temporal pattern of response will be obtained as well. Thus, responses may vary both with concentration and time. The additional complication of temporal organization of response makes the observance of response in real time essential. Responses may be temporally complex under these circumstances and, without visualization of the time course of response, steady states may be impossible to measure. Stop-time experiments with no knowledge of the kinetics of response clearly could be very dissimulating.

One of the theoretical disadvantages of stop-time techniques such as reporter genes is the fact that the kinetics of response production and the reporting of that response production may affect the magnitude of the response. Specifically, the time course of a typical first-order response is given by:

$$\rho_t = \rho_e(1 - e^{-(k([A]+K_A)t)}) \quad [9]$$

in which ρ_e is the response at equilibrium, [A] is the concentration of agonist, K_A is the equilibrium dissociation constant of the agonist-receptor complex, and k is the rate of onset. For stop-time reporter assays, the reaction is allowed to progress for a specified amount of time, and then it is stopped. The amount of product formed (which corresponds to the area under the curve describing ρ_t as a function of time), corresponds to the magnitude of the receptor stimulus (i.e., cAMP). The area under the first order rate of onset curve is given by the integral of equation 9 (Kenakin, 1993b):

$$R_t = R_e\, t + \frac{e^{-k([A]/K_A+1)t}}{k([A]/K_A + 1)} - \frac{1}{k([A]/K_A + 1)} \quad [10]$$

If the relative amount of product formed by two drugs of radically different rates of onset are measured in this manner, it is possible that the agonist of slower onset may appear to be of artificially low potency if the reaction is stopped too quickly. This suggests that longer time points for reporter assays done in stop-time format may reduce artifacts attributable to kinetics.

The issue of real time versus stop time becomes important when agonists produce complex transient responses. Whereas waveforms can be visualized in real time and appropriate decisions made as to where response will be measured, no such luxury always is afforded to stop-time experiments. In these, the reaction between agonist and receptor is terminated at some point and the product of the reaction assayed. If the response is transient, then differences between the total amount of product formed by low concentrations of agonist (which may produce sustained responses) may bear little relationship to the product formed by higher concentrations. Such behavior should be detectable by multiphase dose-response curves. Similarly, the study of constitutive receptor activity with stop-time assays must show that constitutive activity is still actively present at the time of exposure to inverse agonist. Because stop time assays measure a historical response (as in the case of the luciferase reporter gene assay), it may be that the observed response was a burst of constitutive activity that had desensitized back to zero by the time the inverse agonist was added. Under these circumstances, no inverse agonism would be seen. Similarly, if the cell line is very constitutively active, then the diminution in reaction product produced by a short exposure time to inverse agonist may be insignificantly small when compared with the historical reporter response produced by the assay before addition of drug.

2. *Quantitative techniques for functional classification.* The functional classification of drugs and receptors is achieved by the measurement of equilibrium dissociation constants of agonists and antagonists at receptors. There are theoretical and practical reasons to be cautious about considering classification of receptors with agonists.

One of the most common measures is the relative potency of agonists, because this is thought to reflect their affinity and efficacy, both chemical terms unique to the drug and the receptor. However, efficacy is a term specifically related to the agonist, receptor and G-protein (i.e., the γ and δ terms in the cubic ternary complex model); therefore, if receptors couple to different G-proteins in different cellular hosts, then the efficacy of the agonists will differ as well (Kenakin, 1989; fig. 19).

Another common means of classification with agonists is the measurement of agonist affinity by receptor alky-

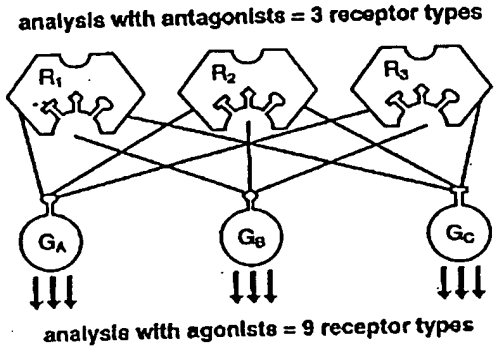


FIG. 19. Schematic diagram of three receptors interacting with three G-proteins. Analysis with antagonists would delineate three receptors, whereas analysis with agonists could define as many as nine 'receptor types.' From Kenakin (1989).

lation (method of Furchgott, 1966). As with potency ratios, the influence of G-protein can be confounding with this technique as well. Specifically, this method may yield an artifactually high measure of agonist potency that is increased by the 'distribution' of the receptor between the G-protein coupled and uncoupled states (Black and Shankley, 1990; MacKay, 1988; 1990a, b). Because the augmentation factor depends upon the degree of distribution which, in turn, depends upon the relative stoichiometry of the receptors and G-proteins, the cellular host system may impose an overriding influence on the resulting estimates of affinity.

The measurement of true neutral antagonist affinity is the most straightforward means of determining chemical terms with which drugs and receptors can be classified. For functional studies, the determination of affinity values by Schild analysis is by far the most powerful method to do this (Kenakin, 1992). This technique is most widely used in isolated tissue studies but also can be applied easily to binding and biochemical studies. Thus, the equilibrium dissociation constant of the antagonist (K_B) should be related to the equiactive dose-ratio of the agonist (determined in the presence and absence of antagonist) by the relationship (Arunlakshana and Schild, 1959):

$$\log (dr\text{-}1) = \log [B] - \log K_B \qquad [11]$$

Thus, various dose-ratios (dr) are plotted as a regression as log (dr-1) upon the logarithms of the molar concentrations of antagonist to yield a straight line of slope unity and intercept pK_B ($-\log K_B$). In general, the Schild method inspects the interaction of an agonist and antagonist over a concentration range and tests the assumption that they have singular properties (i.e., receptor activation for the agonist and receptor occupancy for the antagonist) in a single homogeneous receptor population. These stringent criteria can be a useful tool in themselves as deviation of Schild regression from ideal behavior can be used to detect nonequilibrium steady states such as agonist degradation (Furchgott, 1972), antagonist degradation (Kenakin and Beek, 1987a), temporal inequilibrium (Kenakin, 1980) and the presence of heterogeneous receptor populations (Furchgott, 1972; Kenakin, 1982, 1992). This latter application of Schild analysis can be extremely useful operationally, as general methods to detect mixtures of receptor populations can be derived from Schild analysis in functional studies (Kenakin, 1992). It is well established that Schild analysis is amenable to functional studies in isolated tissues and also can be used in similar approaches in cell cultures (i.e., Gudermann et al., 1993b; Poyner et al., 1992; De Vivo and Maayani, 1986).

An extension of Schild analysis and the additive dose-ratio technique (Paton and Rang, 1965) is a powerful approach to the cancellation of secondary drug properties and the resulting measurement of receptor parameters. Termed *resultant analysis* (Black et al., 1986), this method essentially applies the Schild technique to allow the measurement of dose-ratios under conditions in which the antagonist with suspected secondary actions ($[B_{test}]$) is used in conjunction with a reference antagonist ($[B_{ref}]$). The resulting summated antagonism can be analyzed to yield the contribution of each antagonist (with resulting pK_B estimates). Because the antagonist with the secondary properties is present throughout the analysis, the effects of the secondary properties cancel under the null conditions of the experiment. Thus, Schild regressions for the reference antagonist in the absence and presence of various concentrations of test antagonist are obtained, and the dextral displacement of these regressions can be related to the equilibrium dissociation constant of the test antagonist (Black et al., 1986; for examples see Leff and Morse, 1987; Kenakin and Beek, 1987b; Kenakin and Boselli, 1989)

IX. Mutation of 7TM Receptors

There is increasing evidence that within the field of 7TM receptor research exists a subpopulation of potentially very useful drug targets, namely mutant 7TM receptors. There is also evidence to suggest that the inactive form of 7TM receptors may be a special conformation aimed at keeping inaccessible amino acid sequences that automatically activate G-proteins (Lefkowitz et al., 1993). Existing studies suggest that many man-made mutants of 7TM receptors are spontaneously active (vide infra). In addition, there is increasing evidence that spontaneous 7TM receptors in nature can lead to constitutively active basal responses and thus be associated with human pathology. Some examples of where 7TM receptor mutation can lead to constitutive activity: melanocyte stimulating hormone (Robbins et al., 1993), rhodopsin (Robinson et al., 1992), β_2-adrenergic (Samama et al., 1993), α_2-adrenergic (Ren et al., 1993), leutinizing hormone (Shenker et al., 1993), and thyrotropin (Parma et al., 1993). There are certain diseases that are associated with 7TM receptor mutation; some of these are given in table 11.

There are selected instances in which constitutively active mutant receptors may be associated with disease states: for example, the severe ligand-independent hypercalcemia and hypophosphatemia associated with Jansen-Type metaphyseal chondrodysplasia may relate to a mutant constitutively active PTH receptor (Schipani et al., 1995). Still, it is not clear to what extent the constitutive 7TM receptor activity in general is relevant to human disease (Clapham, 1993; Milligan et al., 1995b). However, the chronic elevation of second-messengers in cells (for example, by constitutive G-protein activity, Lyons et al., 1990; Weinstein et al., 1990; see review by Spiegel et al., 1993) has been shown to lead to cell transformation. In accordance with these findings is the fact that receptor genetic material can function as an

TABLE 11
7TM Receptor mutants in disease

Color blindness	Nathans et al., 1989
Night blindness	Dryja et al., 1993
Retinal degeneration	Robinson et al., 1992
Familial male precocious puberty	Shenker et al., 1993
Familial glucocorticoid deficiency	Clarke et al., 1993
Thyroid adenoma	Parma et al., 1993
Nephrogenic diabetes insipidus	Rosenthal et al., 1993
Neonatal hyperparathyroidism	Pollak et al., 1993
Hypocalcemia	Pollack et al., 1994
Genetic obesity	LaNoue and Martin, 1994
Multigenic Hirschsprung's disease	Puffenberger et al., 1994
Jansen-type metaphyseal chondrodysplasia	Schipani et al., 1995
Nocturnal asthma	Turki et al., 1995
Schizophrenia	Van Tol et al., 1992

agonist-dependent pro-oncogene (Julius et al., 1989; Gutkind et al., 1991; Allen et al., 1991). For example, transfection of functional 5-HT_{1C} receptors into NIH 3T3 cells leads to cell transformation, and injection of the transformed loci into nude mice leads to generation of tumors (Julius et al., 1989). Similarly, fibroblasts transfected with α_{1B}-adrenergic receptors have been shown to be tumorogenic when injected into nude mice (Kim et al., 1994). Agonist *independent* proto-oncogene activity has been observed with transfection of mutant α_1-adrenergic receptors (Allen et al., 1991).

Receptor mutation may play a larger role in the pathology of disease states than thought previously (Pearce and Trump, 1995). Molecular biology techniques such as 'mutational analysis' now are being used to link sequence variations in genes to pathological conditions (Gejman and Gelernter, 1993). For example, there is considerable evidence of polymorphism in human dopamine receptors (Inoue et al., 1993). A link in receptor mutation and disease has been proposed for the dopamine receptor in view of the observed functional polymorphism within the dopamine D_4 receptor gene (Van Tol et al., 1992) and D_3 receptor gene (Nimgaonkar et al., 1993). Significant polymorphic variation in the human population has been detected for dopamine D_4 receptors; this variation may be related to responsiveness to antipsychotic treatment (Van Tol et al., 1992). Similarly, George et al. (1993) found that the dopamine D_4 receptor genotype in 72 patients with chronic alcoholism was heterogeneous, with individuals being homozygous and others heterozygous for the various D_4 receptor alleles. Mutations of dopamine D_4 receptors also appear to be relevant to patients with psychosis, in whom it was found that variation of the gene encoding this receptor conferred susceptibility to delusional disorders (Catalano et al., 1993). Although evidence that some central nervous system diseases may be related to such polymorphism (i.e., psychosis, Catalano et al., 1993; alcoholism, George et al., 1993), there is still debate as to the relevance of this finding to diseases such as schizophrenia (Catalano et al., 1993; Barr et al., 1993).

An interesting polymorphic loci within the coding of the β_2-adrenergic receptor has been discovered that produces mutant receptors (Green et al., 1994). One of these (GLY16) demonstrates accelerated receptor down-regulation and is overrepresented in patients with nocturnal asthma, suggesting a possible correlation and mutant receptor drug target (Turki et al., 1995).

X. Conclusions

This review cites many papers describing the effects of drugs on 7TM receptors that have been expressed in surrogate cell systems. In many of these papers, the receptor antagonist profiles are uniform, high affinity agonist binding is observed, and a functional readout for receptor-mediated response can be obtained. There are also instances in which abnormal stoichiometry and/or the presence or absence of an essential component of the receptor system produces an aberrant behavior that does not reflect therapeutically relevant drug activity. It may be that the expression of 7TM receptors into foreign cells may not always faithfully reflect wild type receptor activity because of the intrinsic organization of cell systems (Neer, 1995). Cells may have optimal ratios of receptors and G-proteins to function (Moghe and Tranquillo, 1995), and the disturbance of these ratios may introduce confounding variables into experiments aimed at measuring drug activity. What is clear is that molecular biology has spawned a renaissance in receptor research, because now these systems can be manipulated in ways never before possible. The use of these new technologies no doubt will aid both in the understanding of drug-receptor mechanisms and the finding of new drug entities.

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APPENDIX E

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Expression of a Cloned P_{2Y} Purinergic Receptor that Couples to Phospholipase C

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SUMMARY

P_{2Y} purinergic receptors previously have been shown to couple either to activation of phospholipase C through a pertussis toxin-insensitive mechanism or to inhibition of adenylyl cyclase through pertussis toxin-sensitive members of the G_i family of G proteins. These and other pharmacological data strongly suggest that multiple P_{2Y} purinergic receptors exist. Webb *et al.* [*FEBS Lett.* 324:219–225 (1993)] cloned a cDNA that, when expressed in frog oocytes, displayed the general pharmacological characteristics of a P_{2Y} purinergic receptor but whose second messenger linkage was not resolved. We have now cloned the meleagrid (turkey) homologue of the previously cloned chick P_{2Y} purinergic receptor and have stably expressed it in a heterologous human cell line (1321N1 astrocytoma cells) to establish its signaling properties. The purinergic receptor agonist 2-methylthio-ATP (2MeSATP) stimulated a marked activation of phospholipase C in 1321N1 cells stably expressing the meleagrid receptor. The order of potency of a series of analogues of ATP and ADP for stimulation of phospholipase C by the receptor expressed in 1321N1 cells [2MeSATP = 2-methylthio-ADP > adenosine 5′-*O*-(2-thio)diphosphate > ADP > 2-chloro-ATP = adenosine 5′-*O*-(3-thio)triphosphate ≥ ATP > adenylyl-imidodiphosphate > UTP] was similar to that observed for P_{2Y} purinergic receptors in turkey erythrocytes and many other tissues and was markedly different from those of the P_{2U} and P_{2X} purinergic receptor subtypes. Stimulation of inositol lipid hydrolysis by P_{2Y} purinergic agonists was not affected by preincubation of cells with pertussis toxin. In contrast to its marked effects on phospholipase C activity, 2MeSATP caused only a small and variable inhibition of cAMP accumulation. Ribonuclease protection analysis of turkey tissues showed that this P_{2Y} purinergic receptor is most highly expressed in blood and brain. Taken together, these results indicate that a phospholipase-C-activating P_{2Y} purinergic receptor has been cloned and stably expressed in 1321N1 astrocytoma cells.

Extracellular adenine nucleotides interact with cell surface receptors to produce a broad range of physiological responses, and multiple receptors that recognize ATP, ADP, and synthetic analogues of these nucleotides have been described (1). These include the P_{2Y} and P_{2X} purinergic receptors, which originally were delineated in studies on smooth muscle responses, the P_{2T} purinergic receptor, which is an ADP-activated receptor on thrombocytes, the P_{2Z} purinergic receptor, which serves a non-selective pore-forming function, and the P_{2U} purinergic receptor, which is widely distributed on a variety of cell types.

Delineation of P_2 purinergic receptors has depended almost entirely on differential selectivities of analogues of ATP and ADP. 2MeSATP is a potent P_{2Y} purinergic receptor agonist, but it is not an effective agonist at P_{2X} and other purinergic receptors (2). There is strong evidence that multiple subtypes exist within several, if not all, of the classes of P_2 purinergic receptors. For example, responses to a broad range of adenine nucleotide analogues have been compared in four tissues expressing P_{2Y} purinergic receptors and three tissues expressing P_{2X} purinergic receptors (3, 4). Many of these analogues showed selectivity or specificity for certain of the P_{2Y} or P_{2X} purinergic receptor responses, suggesting that subtypes of each of these P_2 purinergic receptor types exist.

Subtypes of receptors within a given class often possess very different second messenger-coupling specificities. For example, M_1 muscarinic cholinergic and α_1-adrenergic receptors activate the inositol lipid signaling cascade through G_q family G proteins and phospholipase C, whereas M_2 muscarinic cholinergic and α_2-adrenergic receptors couple through G_i to inhibit adenylyl cyclase (5, 6). Similar contrasts can be seen for subtypes of many other G protein-linked receptors, e.g., receptors for serotonin, angiotensin, endothelin, and thromboxanes. When receptor density achieved in heterologous systems is comparable to receptor density in native tissues, selectivity in G protein/effector coupling is maintained with great fidelity for these receptor subtypes irrespective of the tissue in which they

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ABBREVIATIONS: 2MeSATP, 2-methylthio-ATP; 2ClATP, 2-chloro-ATP; 2MeSADP, 2-methylthio-ADP; α,βMeATP, α,β-methylene-ATP; β,γMeATP, β,γ-methylene-ATP; ADPβS, adenosine 5′-*O*-(2-thio)diphosphate; App(NH)p, adenylyl-imidodiphosphate; ATPγS, adenosine 5′-*O*-(3-thio)triphosphate; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; TCA, trichloroacetic acid; CHO, Chinese hamster ovary; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

are expressed. We have taken advantage of this property of G protein-linked receptors to provide evidence for the existence of at least two P_{2Y} purinergic receptor subtypes.

The prototypical response to P_{2Y} purinergic receptor activation in various tissues is stimulation of inositol lipid hydrolysis (7–11). However, ATP and ADP also decrease cAMP levels in various tissues (12–15), and we recently reported that the pharmacological selectivity of a series of agonists for inhibition of adenylyl cyclase in C6 glioma cells is consistent with that of a P_{2Y} purinergic receptor (16). These effects on cAMP accumulation are blocked by pertussis toxin, which indicates that coupling is through a G protein of the G_i class. Activation of this P_{2Y} purinergic receptor on C6 glioma cells has no effect on inositol lipid hydrolysis or Ca^{2+} mobilization. These results strongly support the idea that at least two P_{2Y} purinergic receptor subtypes exist, one that couples through G_i to inhibit adenylyl cyclase and another that activates phospholipase C through G proteins of the G_q family.

Unambiguous definition of receptor subtypes necessitates association of pharmacological and second messenger signaling properties with receptor proteins whose amino acid sequences have been deduced by molecular cloning. The sequences of only two P_2 purinergic receptors have been reported to date. A P_{2U} purinergic receptor, which is activated by ATP, UTP, and ATPγS, was cloned (17), and Webb *et al.* (18) have reported the sequence of a receptor cloned from chick brain cDNA that when expressed in frog oocytes confers a slowly developing, ATP-stimulated, Ca^{2+}-activated, inward current. Although limited drug concentrations were tested, this receptor displayed the general pharmacological selectivity of a P_{2Y} purinergic receptor. Whether this receptor represents a G_i/adenylyl cyclase-linked or a G_q/phospholipase C-linked receptor was not established, and therefore its relationship, if any, to the putative subtypes of P_{2Y} purinergic receptors that have been identified on the basis of second messenger coupling responses is not clear. Based on this uncertainty as well as the limitations of obtaining detailed pharmacological data in studies of receptors expressed in oocytes, we have cloned the meleagrid (turkey) homologue of the previously cloned chick P_{2Y} purinergic receptor and have stably expressed it in 1321N1 human astrocytoma cells. Activation of this receptor resulted in stimulation of phospholipase C through a pertussis toxin-insensitive pathway, and little or no effect on adenylyl cyclase was observed. Pharmacological analyses of the expressed receptor indicate that it displays drug selectivities that are remarkably similar to those previously observed for P_{2Y} purinergic receptors in a broad range of tissues.

Experimental Procedures

Materials. ATP, ADP, ADPβS, ATPγS, App(NH)p, and UTP were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN); 2MeSATP, 2MeSADP, α,βMeATP, β,γMeATP, and 2ClATP were obtained from Research Biochemicals Inc. (Natick, MA). pcDNA3 is an expression vector developed by Invitrogen (San Diego, CA). pBS SK⁻ is a DNA plasmid vector from Stratagene (La Jolla, CA). G418 sulfate, DMEM, and α-minimum essential medium were obtained from GIBCO/BRL (Grand Island, NY). Fetal bovine serum was obtained from Hyclone Laboratories Inc. (Logan, UT). TCA was obtained from Fisher (Fair Lawn, NJ). 1-Isobutyl-3-methylxanthine, LiCl, isoproterenol, carbachol, and apyrase type IV (EC 3.6.1.5) were obtained from Sigma Chemical Co. (St. Louis, MO). Forskolin [as the water-soluble analogue 7β-deacetyl-7β-(γ-*N*-methylpiperazino)butyryl-forskolin] was obtained from Calbiochem (La Jolla, CA). Pertussis toxin was obtained from List Biological Laboratories Inc. (Campbell, CA). Restriction enzymes were obtained from Pharmacia LKB Biotechnology (Piscataway, NJ). The Sequenase 2.0 kit was obtained from United States Biochemical (Cleveland, OH). The RPAII kit was obtained from Ambion Inc. (Austin, TX).

Cloning of turkey brain receptor. Total RNA from turkey brain was isolated by a modification of the method of Chomczynski and Sacchi (19). Briefly, frozen turkey brain (1 g of tissue) was homogenized in guanidinium thiocyanate, 2-mercaptoethanol, 0.3 M sodium acetate, pH 5.2, and extracted with acid phenol. After addition of chloroform to separate the two phases, RNA was precipitated from the aqueous phase with 2 volumes of ethanol. Total RNA (1 μg) was then reverse transcribed with dT_{17} as a primer for Moloney murine leukemia virus reverse transcriptase, using the Perkin Elmer/Cetus GeneAmp RNA kit. Two oligonucleotide primers based on the chick brain P_{2Y} purinergic receptor sequence (18) were used to amplify the turkey brain clone. The upstream primer (5′-GAGAGGATCCATCATGACCGA-AGCCCTCAT-3′) included a *Bam*HI site, the last three bases of the 5′ noncoding region, and the first 17 bases of the coding region of chick brain P_{2Y} purinergic receptor. The downstream primer (5′-TCTCTCTAGATCACAAACTGGTGTCCCCGTT-3′) included the last 18 bases of the chick brain P_{2Y} purinergic receptor coding region, a stop codon, and an *Xba*I site. The conditions for PCR amplification were 95° for 1 min and 55° for 1 min, repeated for 35 cycles. The resulting amplified cDNA was cloned into pcDNA3 and then subcloned into M13 vectors in both directions. The clones were sequenced on both strands using the Sequenase 2.0 kit and primers based on the chick brain P_{2Y} purinergic receptor sequence. The sequence of turkey brain P_{2Y} was confirmed by sequencing single clones from four separate PCR amplifications.

Transfection of mammalian cells. pcDNA3 expression vector contains a cytomegalovirus promoter for high expression levels in mammalian cells and a neomycin resistance gene for continual selection of expressing cells. Cells were transfected by the calcium phosphate precipitation method of Chen and Okayama (20). 1321N1 human astrocytoma cells were plated at a density of 1×10^6 cells/plate in 100-mm tissue culture plates and allowed to attach overnight. pcDNA3 plasmid DNA with or without receptor coding sequence was suspended in calcium phosphate buffer and incubated overnight with 1321N1 cells at 35° in 3% CO_2. The cells were washed twice with growth medium and grown for 48 hr before subculturing and selection with G418 sulfate. G418-resistant cells were subcloned by isolation and expanded, and 12–18 clones were screened for expression of the P_{2Y} purinergic receptor.

Tissue culture. 1321N1 human astrocytoma cells were grown in monolayer culture at 37° in 5% CO_2 in high-glucose DMEM supplemented with 5% fetal bovine serum. Transfected cells were maintained in medium supplemented with 900 μg/ml G418 sulfate. The growth medium was changed every fourth day and cells were subcultured at a density of 1×10^5 cells/ml of medium; postconfluent cells were used for assay on day 7.

Inositol phosphate accumulation. Inositol phosphate accumulation was determined as described previously (16), with the following exceptions. Cells were labeled overnight with 1 μCi/ml [^{3}H]inositol in inositol-free DMEM containing 5% dialyzed fetal bovine serum. Fetal bovine serum was dialyzed against 4 liters of Earle's salts changed three times over 48 hr. Earle's salts are 1.8 mM $CaCl_2$, 5.3 mM KCl, 0.8 mM $MgSO_4$, 117 mM NaCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, and 5.6 mM glucose, pH 7.4. Labeled cells were washed once, the medium was replaced with 890 μl of 20 mM HEPES-buffered Eagle's medium, pH 7.4, without LiCl, and the cells were maintained in a 37° water bath for 30 min before proceeding. This step was necessary to reduce background levels of [^{3}H]inositol phosphates, so that agonist-stimulated accumulation could be detected more easily. After incubation for 30 min at 37° in [^{3}H]inositol-free medium, 10 μl of LiCl were added to prelabeled cells to a final concentration of 10 mM and incubation was continued for an additional 10 min. Receptor agonists in 100 μl of medium were added, cells were incubated for 20 min at 37°, and

reactions were terminated by aspiration of the medium and addition of 1 ml of ice-cold 5% TCA. [^{3}H]Inositol phosphates were purified by anion exchange chromatography as described previously (21). [^{3}H] Phospholipids were collected after solubilization of the TCA-precipitated cells with 1 ml of 1 N NaOH.

cAMP accumulation. cAMP accumulation was determined from the conversion of [^{3}H]ATP to [^{3}H]cAMP as described previously (16), with the following exceptions. Transfected 1321N1 cells grown in 12-well plates were incubated overnight with 1 μCi/ml [^{3}H]adenine in DMEM containing 5% dialyzed fetal bovine serum. The cells were washed once with 890 μl of 20 mM HEPES-buffered Eagle's medium, pH 7.4, and incubated at 37° for 30 min before proceeding. Ten microliters of 1-isobutyl-3-methylxanthine dissolved in dimethylsulfoxide were then added to a final concentration of 200 μM and incubation was continued for an additional 10 min. Receptor agonists in 100 μl of medium were added, cells were incubated for 5 min at 37°, and the reactions were terminated by aspiration of the medium and addition of 1 ml of ice-cold 5% TCA. [^{3}H]ATP and [^{3}H]cAMP were separated by Dowex and alumina chromatography, as described previously (22).

RNase protection assays. Levels of turkey P_{2Y} purinergic receptor mRNA in various tissues were quantitated by RNase protection assay using the RPAII kit, according to the vendor's protocol. The ribonucleotide probe for the meleagrid P_{2Y} purinergic receptor was generated from a subcloned fragment that comprised nucleotides 654–927 of the coding sequence. Fragments were generated by PCR amplification using gene-specific primers and were then subcloned into pBS SK$^-$. Total RNA from turkey blood was isolated by lysis of freshly washed adult erythrocytes in guanidinium thiocyanate and sedimentation through a cesium chloride cushion as described (23). Total RNA from all other tissues was prepared by a modification of the method of Chomczynski and Sacchi (19), as described above.

Results

Isolation of a P_{2Y} purinergic receptor cDNA clone from turkey brain. We have previously studied in detail a phospholipase C-linked P_{2Y} purinergic receptor on turkey erythrocytes. The recent cloning of a P_{2Y} purinergic receptor from chick brain (18) provided the means for isolation of a turkey homologue of this receptor and allowed the second messenger coupling response and pharmacological specificity of this P_{2Y} purinergic receptor to be determined. To isolate a full length cDNA clone of the meleagrid P_{2Y} purinergic receptor, PCR amplification was performed on total RNA isolated from turkey brain, using oligonucleotide primers identical to the beginning and end of the coding sequence of the chick brain P_{2Y} purinergic receptor. The resulting amplified fragment was cloned and sequenced, and sequence analysis indicated that the meleagrid sequence was >98% identical at the nucleotide level (17 differences in 1089 nucleotides) to the chick gene.[1] The only amino acid difference between the two genes was a conservative substitution of threonine-28 to serine. Amplification of a genomic clone with the same primers resulted in a fragment identical in size to the cDNA, suggesting that the P_{2Y} purinergic receptor gene lacks an intron. Given the nearly complete identity of the two sequences, the second messenger coupling and pharmacological specificity of the meleagrid and chick homologues of the P_{2Y} purinergic receptor should be the same. Therefore, the meleagrid sequence was prepared for expression and characterization in eukaryotic cells.

Expression of meleagrid P_{2Y} purinergic receptor in 1321N1 cells and stimulation of inositol phosphate accumulation. pcDNA3 vector alone or vector containing the coding sequence of the meleagrid putative P_{2Y} purinergic receptor was transfected into 1321N1 human astrocytoma cells. These cells express no detectable endogenous receptors for adenine nucleotides (24) and endogenously express an M1 muscarinic cholinergic receptor coupled to stimulation of phospholipase C (21, 25). 1321N1 cells transfected with the P_{2Y} purinergic receptor construct (1321N1-P2Y cells) were cloned and screened for 2MeSATP-stimulated inositol phosphate accumulation or inhibition of cAMP accumulation. Initially, very little stimulation or inhibition of inositol phosphate or cAMP accumulation was observed in any of the transfected cell lines. However, certain of the 1321N1-P2Y cell clones expressed basal levels of [^{3}H]inositol phosphates that were markedly greater than basal levels in the vector-transfected clonal cell lines. We reasoned that this elevated [^{3}H]inositol phosphate accumulation could be due, at least in part, to release of endogenous ATP and/or ADP into the medium, with subsequent activation of an expressed P_{2Y} purinergic receptor and elevation of [^{3}H] inositol phosphates during the 18-hr [^{3}H]inositol labeling period. Two approaches were used in an attempt to decrease basal levels of [^{3}H]inositol phosphates. 1321N1-P2Y cells were treated with apyrase overnight during the [^{3}H]inositol labeling step, with the goal of decreasing ATP and/or ADP levels in the cell medium. Alternatively, [^{3}H]inositol-labeled 1321N1-P2Y cells were washed free of [^{3}H]inositol and incubated for 30 min in the absence of LiCl, to allow elevated levels of [^{3}H]inositol phosphates to decrease.

The data presented in Fig. 1 illustrate the results of the different treatments of pcDNA3 vector-transfected (1321N1-vector) cells and 1321N1-P2Y cells. In wild-type control (data not shown) and vector-transfected cells (Fig. 1A), 10 μM 2MeSATP and other analogues of ATP and ADP (Ref. 24 and data not shown) had no effect on [^{3}H]inositol phosphate accumulation. Activation by 500 μM carbachol of an endogenous M1 muscarinic cholinergic receptor on 1321N1 cells markedly elevated [^{3}H]inositol phosphate levels. There was a 10-fold increase in basal [^{3}H]inositol phosphate levels in cells stably transfected with the P_{2Y} purinergic receptor (Fig. 1B). A variable small increase in [^{3}H]inositol phosphate levels above the high background level occurred in response to 2MeSATP in 1321N1-P2Y cells and the large response to carbachol was maintained. If 1321N1-P2Y cells were switched to [^{3}H]inositol-free medium for 30 min before incubation with LiCl and receptor agonists (Fig. 1C), the basal level of [^{3}H]inositol phosphates was reduced, relative to 1321N1-P2Y cells that did not receive this incubation before addition of LiCl (Fig. 1, compare C and B). A marked 2MeSATP-stimulated accumulation of [^{3}H]inositol phosphates, which was approximately 50% of the level of stimulation observed with carbachol, occurred in these cells. Apyrase treatment during the overnight [^{3}H]inositol labeling step (Fig. 1D) also resulted in 1321N1-P2Y cells with reduced basal levels of radioactivity, relative to untreated 1321N1-P2Y cells (Fig. 1, compare D and B). 2MeSATP markedly stimulated inositol phosphate accumulation in apyrase-treated cells, to levels that exceeded those observed with carbachol. Therefore, when 1321N1-P2Y cells were treated with apyrase overnight or were preincubated for 30 min after a change of medium, the basal accumulation of [^{3}H]inositol phosphates was decreased and 2MeSATP-stimulated [^{3}H]inositol phosphate accumulation was readily detected. We preferred, in subsequent experiments, to decrease basal [^{3}H]inositol phosphate levels by incubating cells for 30 min after a change to [^{3}H]inositol-free

[1] The nucleotide sequence of the meleagrid P_{2Y} purinergic receptor has been submitted to the GenBank database with accession number U09842.

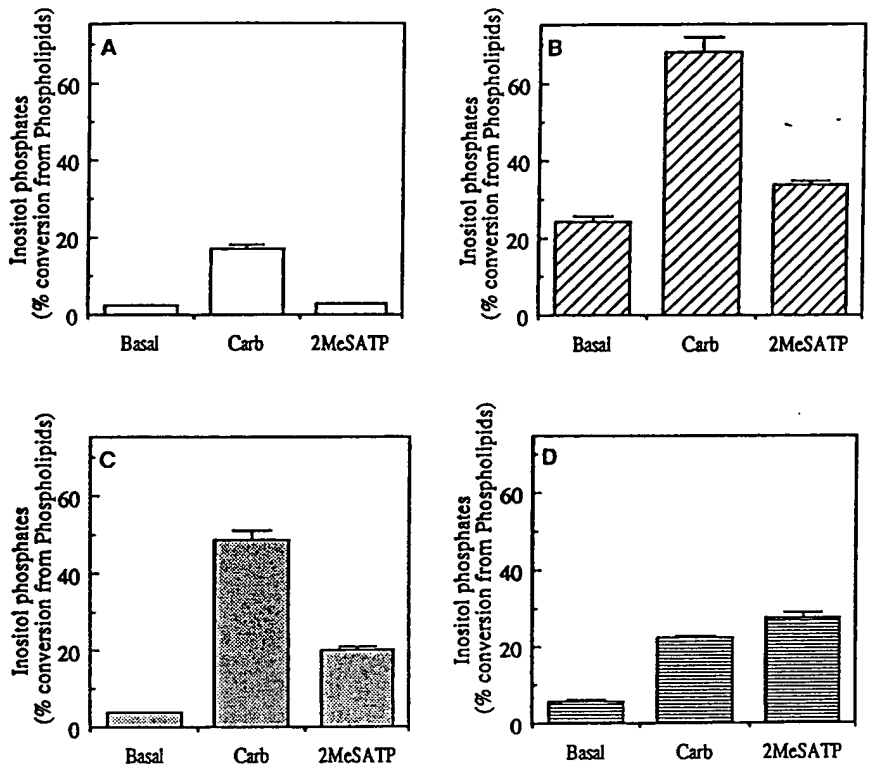

Fig. 1. Effects of preincubation and apyrase treatment on basal and agonist-stimulated levels of inositol phosphate accumulation in vector- and P_{2Y} purinergic receptor-transfected 1321N1 cells. A and B, 1321N1-vector (A) and 1321N1-P2Y (B) cells were treated overnight with [^{3}H]inositol and assayed for [^{3}H]inositol phosphate accumulation without a 30-min preincubation. C, 1321N1-P2Y cells were assayed for [^{3}H]inositol phosphate accumulation after a 30-min preincubation, as described in Experimental Procedures. D, 1321N1-P2Y cells were treated overnight, during [^{3}H]inositol labeling, with apyrase (2 units/ml) and were then assayed for [^{3}H] inositol phosphate accumulation. Data shown are mean ± standard deviation for a representative experiment assayed in quadruplicate. Similar results were obtained in two other experiments. *Carb*, carbachol.

medium instead of incubating cells overnight with apyrase, to avoid any potential hydrolytic effect of apyrase on the various adenine nucleotides used as receptor agonists. Incubation of 1321N1-vector cells overnight with apyrase or switching of these cells to [^{3}H]inositol-free medium before incubation with LiCl and agonists did not result in appearance of a 2MeSATP-stimulated response (data not shown).

Pharmacological characterization of the expressed meleagrid receptor. To characterize more fully the putative P_{2Y} purinergic receptor from turkey brain, inositol phosphate accumulation was studied with several P_{2Y}-, P_{2U}-, and P_{2X}-selective and nonselective agonists. As anticipated from the almost complete sequence identity with the previously cloned chick P_{2Y} purinergic receptor, the meleagrid receptor showed the pharmacological profile expected of a P_{2Y} purinergic receptor (Fig. 2; Table 1). Adenine nucleotides previously shown to be effective P_{2Y} purinergic receptor agonists were all full agonists at the expressed receptor, with little variation in the maximal response obtained within an experiment. 2MeSATP and 2MeSADP, which are agonists that were previously shown to be selective for P_{2Y} purinergic receptors (2, 3, 10, 16), showed the highest potency for stimulation of inositol phosphate accumulation in 1321N1-P2Y cells. The P_{2X} purinergic receptor-selective agonists α,βMeATP and β,γMeATP did not stimulate inositol phosphate accumulation in 1321N1-P2Y cells. Furthermore, UTP had little effect, indicating that the expressed receptor is not a P_{2U} purinergic receptor subtype. The pharmacological profile obtained for stimulation of inositol phosphate accumulation in 1321N1-P2Y cells [2MeSATP = 2MeSADP > ADPβS > ADP > 2ClATP = ATPγS ≥ ATP > App(NH)p > UTP] was very similar to that obtained for the P_{2Y} purinergic receptor coupled to inhibition of adenylyl cyclase activity in rat C6–2B glioma cells (Table 1). With the exception of the potencies of ADP and 2ClATP, the pharmacological profile of the expressed receptor was similar to that previously

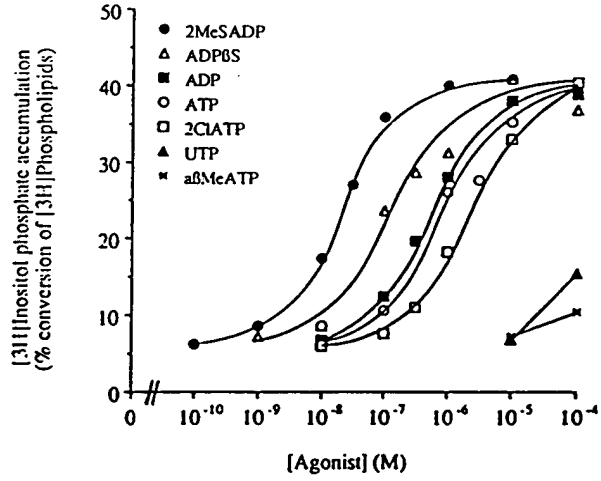

Fig. 2. Concentration-effect curves for purinergic receptor agonists in P_{2Y} purinergic receptor-transfected 1321N1 cells. Inositol phosphate accumulation was measured in [^{3}H]inositol-prelabeled 1321N1-P2Y cells that had been subjected to a 30-min preincubation in [^{3}H]inositol-free medium, as described in Experimental Procedures. Concentration-effect curves were generated by treating cells for 20 min with varying concentrations of agonist. *Curves* represent mean data obtained from a single experiment assayed in quadruplicate. Similar results were obtained in two or three other experiments.

obtained for P_{2Y} purinergic receptors coupled to phospholipase C in turkey erythrocyte membranes (4, 10).

P_{2Y} purinergic receptors previously were shown to couple through a pertussis toxin-insensitive mechanism to activation of phospholipase C or through pertussis toxin-sensitive members of the G_i family of G proteins to inhibition of adenylyl cyclase. Stimulation by either 2MeSATP or carbachol of inositol phosphate accumulation in 1321N1-P2Y cells was not sensitive to pretreatment of cells with 100 ng/ml pertussis toxin (Table 2). This concentration of pertussis toxin previ-

TABLE 1

Relative potencies of purinergic receptor agonists for activation of P_{2Y} purinergic receptors

Inositol phosphate accumulation was measured in 1321N1-P2Y cells as described in Experimental Procedures. Concentration-effect curves were generated by incubating [^{3}H]inositol-labeled cells for 20 min with varying concentrations of agonist, as illustrated in Fig. 2. $K_{0.5}$ values were calculated as the concentration of agonist required to produce a half-maximal effect, as determined by nonlinear regression analysis. Data shown are mean ± standard error of $K_{0.5}$ values for three or four independent experiments.

Agonist	$K_{0.5}$		
	1321N1-P2Y	C6-2B[a]	Turkey erythrocyte[b]
		nM	
2MeSATP	30.5 ± 14.8	3.9 ± 1.3	8 ± 2
2MeSADP	25.7 ± 9.6	10.0 ± 5.3	6 ± 3
ADPβS	233 ± 76	675 ± 118	96 ± 27
ADP	534 ± 61	3,000 ± 580	8,000 ± 2,000
ATPγS	2,790 ± 330	3,166 ± 1,014	1,260 ± 380
2ClATP	2,990 ± 1,320	2,140 ± 761	72 ± 19
ATP	4,120 ± 1,970	9,000 ± 1,703	2,800 ± 700
App(NH)p	>10,000	>10,000	4,450 ± 1,150
UTP	>10,000	>10,000	143,000 ± 44,000
αβMeATP	NE[c]	NE	>100,000
βγMeATP	NE	NE	>100,000

[a] Values for P_{2Y} receptor-promoted inhibition of cAMP accumulation in rat C6-2B glioma cells, obtained from Boyer *et al.* (16).

[b] Values for P_{2Y} receptor-mediated activation of phospholipase C in turkey erythrocyte membranes, obtained from Burnstock *et al.* (4)

[c] NE, no effect at 100 μM.

TABLE 2

Effect of pertussis toxin treatment on receptor-stimulated inositol phosphate accumulation in 1321N1-P2Y cells

1321N1-P2Y cells were treated for 18 hr with vehicle (control) or with 100 ng/ml pertussis toxin. The cells were washed, preincubated for 30 min in [^{3}H]inositol-free medium, and assayed for inositol phosphate accumulation in the absence of added drug (basal) or in the presence of 500 μM carbachol or 10 μM 2MeSATP. [^{3}H] Inositol phosphate accumulation was quantitated as described in Experimental Procedures, and the data are mean ± standard error for three experiments assayed in quadruplicate.

	[^{3}H]Inositol phosphate accumulation		
	Basal	Carbachol	2MeSATP
	% conversion		
Control	16.1 ± 1.3	66.6 ± 15.0	49.4 ± 7.0
Pertussis toxin	17.1 ± 3.3	69.7 ± 17.6	51.0 ± 5.8

ously was shown to cause full ADP-ribosylation of pertussis toxin-sensitive G proteins in 1321N1 cells and to completely block G_i-mediated inhibition of adenylyl cyclase (26, 27).

In addition to activation of phospholipase C, P_{2Y} purinergic receptors previously were shown to inhibit adenylyl cyclase. Our data with C6 glioma cells suggest that these two different second messenger responses occur through activation of two different P_{2Y} purinergic receptor subtypes (16). However, this remains to be formally proven. Thus, it was important to determine whether the cloned P_{2Y} purinergic receptor coupled to adenylyl cyclase in addition to phospholipase C. cAMP levels were elevated by isoproterenol-mediated stimulation of the β-adrenergic receptors endogenously expressed on 1321N1 cells. Incubation of 1321N1-P2Y cells with isoproterenol plus 2MeSATP resulted in a slight increase in cAMP accumulation over that observed with isoproterenol alone (Fig. 3). This increase may be secondary to inositol-1,4,5-trisphosphate-promoted Ca^{2+} mobilization and Ca^{2+}-mediated stimulation of adenylyl cyclase activity. Similar results were previously observed during stimulation of 1321N1 cells with carbachol (28, 29). To fully activate adenylyl cyclase activity, 1321N1-P2Y cells were incubated with isoproterenol plus forskolin. Data

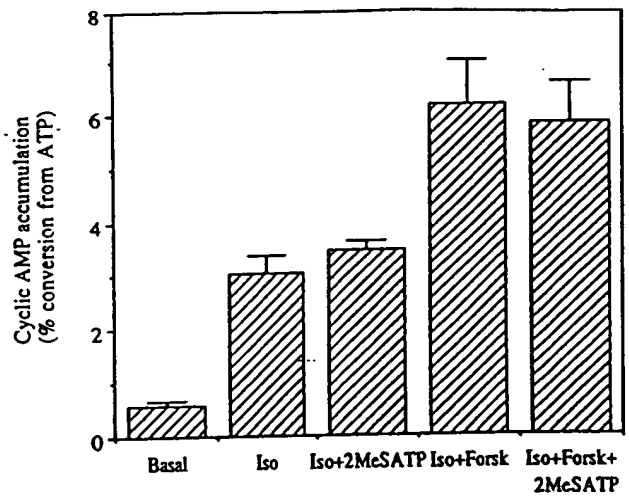

Fig. 3. Absence of 2MeSATP-promoted inhibition of cAMP accumulation in P_{2Y} receptor-transfected 1321N1 cells. 1321N1-P2Y cells were prelabeled with [^{3}H]adenine overnight and subjected to a 30-min incubation in [^{3}H]adenine-free medium, as described in Experimental Procedures. The labeled and washed cells were then incubated for 5 min in the absence of added drug (*Basal*) or in the presence of 10 μM isoproterenol (*Iso*), 10 μM isoproterenol plus 10 μM 2MeSATP (*Iso+2MeSATP*), 10 μM isoproterenol plus 10 μM forskolin (*Iso+Forsk*), or 10 μM isoproterenol plus 10 μM forskolin plus 10 μM 2MeSATP (*Iso+Forsk+2MeSATP*) and were assayed for cAMP accumulation as described in Experimental Procedures. The data shown are mean ± standard error for three to nine experiments assayed in quadruplicate.

averaged from nine experiments showed that 2MeSATP had no effect on isoproterenol- plus forskolin-stimulated cAMP accumulation (Fig. 3). However, in four of the nine experiments a small (20–30%) but significant inhibition of cAMP accumulation occurred. This finding suggests that, whereas stimulation of inositol phosphate accumulation is the primary signaling pathway for this P_{2Y} purinergic receptor, some coupling to inhibition of adenylyl cyclase activity may occur. Desensitization of P_{2Y} purinergic receptors is not responsible for the lack of inhibition, because incubation of 1321N1-P2Y cells overnight with 2 units/ml apyrase before assay did not reveal any inhibition by 2MeSATP of cAMP accumulation (data not shown).

Tissue distribution of the cloned meleagrid P_{2Y} purinergic receptor. RNase protection assays on total RNA isolated from various meleagrid tissues were performed to establish the localization of P_{2Y} purinergic receptor transcripts. An antisense ribonucleotide probe specific for the P_{2Y} purinergic receptor sequence was constructed, and this probe hybridized most strongly with RNA from turkey brain, lung, and blood (Fig. 4). Lesser intensity hybridization was observed with RNA from stomach, gut, and skeletal muscle. With the exception of hybridization levels in lung, these results correlate with data obtained by Webb *et al.* (18) by Northern blot analysis of chick tissues.

Discussion

The relative potencies of a series of 11 adenine nucleotide analogues for stimulation of inositol phosphate accumulation confirm that the meleagrid receptor that has been stably expressed in 1321N1 human astrocytoma cells is a P_{2Y} purinergic receptor (Fig. 2; Table 1). Previous comparative studies of agonist potencies across a number of tissues have led to the suggestion that multiple subtypes of P_{2Y} purinergic receptors

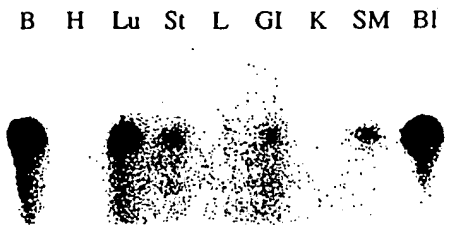

Fig. 4. Tissue distribution of the cloned meleagrid P_{2Y} purinergic receptor. RNase protection assays were performed on 25 μg of total RNA isolated from various meleagrid tissues, using a 274-base pair antisense ribonucleotide probe specific for the cloned meleagrid P_{2Y} purinergic receptor, as described in Experimental Procedures. *B*, brain; *H*, heart; *Lu*, lung; *St*, stomach; *L*, liver; *GI*, gastrointestinal tract; *K*, kidney; *SM*, skeletal muscle; *Bl*, blood.

exist (3, 4). No firm subclassification has been established on the basis of these tissue responses, and it is not yet possible to unambiguously associate the receptor that has been cloned and expressed here with any given set of tissue responses. However, within the limitations of the agonists that have been studied, this P_{2Y} purinergic receptor expresses pharmacological specificities not unlike those of the P_{2Y} purinergic receptor of the guinea pig taenia coli, rat C6 glioma cells, and turkey erythrocytes.

We believe that studies on second messenger responses may more strongly suggest the existence of subtypes of P_{2Y} purinergic receptors than do the differences in agonist potencies that have been observed in tissue responses. Thus, P_{2Y} purinergic receptors have been shown to activate phospholipase C and inhibit adenylyl cyclase. In two model systems that have been studied in detail, the P_{2Y} purinergic receptor couples either to G_i and adenylyl cyclase (16) or to G_{11} and phospholipase C (10), but not to both. Although this does not prove that a similar fidelity of coupling will be observed in all tissues, this is a strong possibility, based on the strict selectivity of coupling to different G proteins and second messenger cascades that has been observed for subtypes in many receptor classes. Expression of the meleagrid receptor in 1321N1 human astrocytoma cells conferred marked responsiveness of phospholipase C to activation by P_{2Y} purinergic receptor agonists. Activation of phospholipase C was completely insensitive to pertussis toxin. These results are consistent with the idea that a P_{2Y} purinergic receptor subtype that couples through a G_q type of G protein to activate phospholipase C has been cloned. In preliminary experiments with CHO cells that were stably transfected with the meleagrid P_{2Y} purinergic receptor, we have observed a similar marked stimulation by P_{2Y} purinergic receptor agonists of inositol phosphate accumulation.[2] This suggests that linkage to phospholipase C is an intrinsic property of this P_{2Y} purinergic receptor subtype and not of the cell line.

A small variable effect of 2MeSATP on the inhibition of cAMP accumulation in transfected 1321N1-P2Y cells was observed in four of nine experiments. Overexpression of the receptor protein in 1321N1 cells may allow the receptor to couple weakly to other G protein subunits and thus produce the variable inhibition of adenylyl cyclase activity that was seen. Such a result was originally observed with overexpression of transfected muscarinic receptors in CHO cells, in which a G_i-coupled m2 muscarinic receptor was found to couple weakly to phospholipase C (30). The physiological importance of such secondary coupling is not known, but the coupling is presumed to be an artifact of the presence of unnaturally high levels of receptor protein. An important next target for molecular cloning will be the G_i-linked P_{2Y} purinergic receptor that exclusively inhibits adenylyl cyclase in C6 glioma cells and in other tissues.

This laboratory has extensively studied a P_{2Y} purinergic receptor linked to activation of phospholipase C on turkey erythrocytes (10, 11, 31–33). Because we have no protein sequence for this receptor, we cannot with certainty equate the meleagrid P_{2Y} purinergic receptor that has been cloned with the erythrocyte signaling protein. However, RNase protection experiments indicate that the meleagrid P_{2Y} purinergic receptor mRNA is found at highest levels in blood and brain. The activation of phospholipase C observed with the receptor expressed in 1321N1 cells is very consistent with the signaling activity of the erythrocyte receptor, and the overall potencies of agonists also closely match. The differences observed in agonist (e.g., 2ClATP and ADP) potencies between the turkey erythrocyte P_{2Y} purinergic receptor and the transfected P_{2Y} purinergic receptor may be due to differences in assay conditions, because phospholipase C activity was measured in erythrocyte membranes rather than in intact cells. Exploration of these differences awaits a means for more direct comparison of the expressed and endogenous P_{2Y} purinergic receptors.

We previously used [^{35}S]ADPβS to label P_{2Y} purinergic receptors on plasma membranes purified from turkey erythrocytes (31). Although a complete understanding has not been obtained, extensive analysis of the turkey plasma membrane [^{35}S]ADPβS binding site with a very broad range of analogues of ATP and ADP has led us to question the validity of the conditions previously reported for putative P_{2Y} purinergic receptor labeling.[3] These caveats notwithstanding, [^{35}S]ADPβS was used in preliminary experiments in an attempt to label P_{2Y} purinergic receptors in transfected cells. No reproducible difference in total [^{35}S]ADPβS binding was observed between control cells and cells expressing the P_{2Y} purinergic receptor construct. This lack of a difference in radioligand binding was observed in both intact cell and membrane binding assays. Because [^{35}S]ADPβS binding was not inhibited by 2MeSATP in either control or P_{2Y} purinergic receptor-transfected cells, no specific binding of the radiolabeled agonist could be detected. The availability of a radiolabeled, high affinity, P_{2Y} purinergic receptor antagonist may be necessary to quantitate P_{2Y} purinergic receptor levels in cells stably expressing this receptor.

The observations described here have several implications in the study of P_2 purinergic receptors. The experiments with apyrase suggest, but do not prove, that release of ATP/ADP occurs from 1321N1 human astrocytoma cells and that this released nucleotide activates the expressed P_{2Y} purinergic receptors. A similar phenomenon has been observed during expression of human P_{2U} purinergic receptors in the same cells (34). The difference in response to 2MeSATP versus carbachol in apyrase-treated cells, compared with the difference in cells not receiving apyrase treatment (Fig. 1, compare D with B and C), suggests that considerable down-regulation of P_{2Y} purinergic receptors may occur as a consequence of released ATP/ADP. Thus, the level of expression of a P_2 purinergic receptor in a given cell likely depends on the extent to which that cell releases adenine nucleotides. A corollary to this could be that the best choice for expression of a P_{2Y} purinergic receptor might be a cell type, e.g., an epithelial cell line such as HT-29 human

[2] T. M. Filtz and T. K. Harden, unpublished observations.

[3] T. K. Harden, unpublished observations.

colon carcinoma cells, that expresses a very active endogenous P_{2U} purinergic receptor (35), or even rat C6 glioma cells, which express a P_{2Y} purinergic receptor that does not activate phospholipase C but, rather, inhibits adenylyl cyclase (16). The possibility also exists that cells not apparently expressing a P_{2Y} or P_{2U} purinergic receptor under normal culture conditions may nevertheless do so if conditions are changed to minimize the effect of released ATP/ADP. For example, an endogenous P_2 purinergic receptor-activated phospholipase C response was revealed in CHO cells that were treated overnight with apyrase.[4]

The meleagrid P_{2Y} purinergic receptor is remarkably similar to the previously cloned chick P_{2Y} purinergic receptor. The single amino acid difference is a conservative substitution that retains a putative glycosylation site near the amino terminus. This conservation of sequence would suggest that the chick homologue is also a phospholipase C-linked purinergic receptor. Purinergic receptors should share characteristics with other members of the G protein-linked receptor superfamily of proteins, including the existence of multiple subtypes linked to different second messenger systems. We believe that additional subtypes of P_{2Y} purinergic receptors, including those linked to inhibition of adenylyl cyclase activity, await isolation and sequencing. A comparison of tissues exhibiting P_{2Y} purinergic receptor-mediated responses (1) with the tissue distribution of transcripts for the cloned P_{2Y} purinergic receptor (Fig. 4 and Ref. 18) reveals a curious lack of P_{2Y} purinergic receptor mRNA in heart. Such results could be suggestive of the presence of another subtype of P_{2Y} purinergic receptor in cardiac tissue.

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